The interaction of invariant chain-derived peptides (CLIP) with mouse class II MHC molecules

Sarah Melanie Weenink

A thesis submitted for the degree of
Doctor of Philosophy of The Australian National University

John Curtin School of Medical Research
Australian National University
Canberra, Australia.

December 1999

This thesis presents the results of research undertaken at the John Curtin School of Medical Research at The Australian National University, Canberra. This work was conducted initially in the Human Genetics Group, Division of Molecular Medicine and, later, in the Antigen Presentation Laboratory, Division of Cell Biology and Immunology. During this time, I was the recipient of an Australian National University postgraduate scholarship.

I hereby declare that this submission is my own original work, except where due acknowledgement has been made in the text. The material presented in this thesis has not been submitted previously for a degree or diploma at this or any other university.

Sarah Melanie Weenink
20 December 1999.

For my parents,
Pat and Brian

ACKNOWLEDGMENTS

It is an incredible feeling to be writing the acknowledgments of my thesis. Of greatest delight, it signifies to me that end is in sight. In addition, it gives me an opportunity to reflect back and remember all the wonderful people who have helped me along the way.

I wish to thank Dr Anand Gautam for establishing this very fruitful research project and for all his enthusiasm and support over the last few years. I thank Dr Peter Milburn for overseeing my preparation of this thesis and I am extremely grateful for his inherent fascination of the properties of the invariant chain protein. I would like to express my thanks to all the members of my academic advisory panel, Professor Bob Blanden, Professor Chris Parish, Dr Arno Müllbacher and Dr Simon Easteal. In particular, I wish to acknowledge Bob and Simon, together with Dr Peter Jeffrey and Professor Phil Board, for their assistance at critical times.

I am tremendously grateful to all the members of the Antigen Presentation Group. I am indebted to Yvonne Gautam for purifying some of the mouse class II MHC proteins, establishing the ELISA protocol in our laboratory and for being of such wonderful help during my running of those assays. I also wish to make particular mention of the support, technical and otherwise, that I have received from Vanda Quinn, Terry Moore, Tanya Boston and Maria Yang. To the sole remaining member of the Antigen Presentation Group, Aparna Bhatnagar, I extend my warmest thanks for her friendship and I wish her a speedy completion of her own doctoral research.

I owe huge thanks to Geoff Osborne and Sabine Grüninger of the JCSMR FACS Unit for their expert help and advice and for always ensuring that I had a good laugh, if not a good result. I would like to thank the staff of the ANU Biomolecular Resource Facility, especially Kerry McAndrew who synthesised the extensive number of peptides used during this research and conducted extensive "font research" on my behalf. I thank Kerry particularly for the many peptides that were always "the last, I promise!". I am very grateful to Dr Anne Cooke of the University of Cambridge, UK, for supplying the M12.NOD, M12.ASP and M12.PRO cell lines.

I have been tremendously lucky to have had the friendship of Erin O'Neill and Alanna Hurne over the last few months. I have been overwhelmed by their selfless generosity and unwavering support even in the midst of their own significant thesis troubles.

I wish to thank the members of the Human and Molecular Genetics Groups and of the Division of Immunology and Cell Biology for their friendship and encouragement. In particular, I offer my heartfelt thanks to Marjorie Coggan, Leanne Langton and Glenys Noble for their kindness and concern. I also wish to thank Lynn Croft, Cathy Gilchrist, Genevieve Herbert, Tony Manderson, Esmee Weil, Cheryl Wise and the staff of both the JCSMR Photography Unit and the Media and Washup Facility.

My special thanks go to my dear friends, Sarah Wade, Soren Blau and Tim Denham for giving me a life beyond the walls of the JCSMR. I also thank Nikki Van Dijk, David Fotheringham, Keith Sircombe and Sofia Levtchenkova for all the wonderful happy memories.

Lastly, I am immensely grateful to my whanau for all their encouragement and patience throughout this endeavour. I wish to dedicate this thesis to my wonderful parents, Pat and Brian Weenink. My debt to them extends well beyond dollars.

ABSTRACT

Class II MHC molecules at the surface of antigen-presenting cells present peptides to $CD4^+$ T lymphocytes. CLIP represents a nested set of peptides derived from the primary interaction site of the class II MHC molecular chaperone, invariant chain (Ii), which binds to many allelic variants, unlike fragments of protein antigen.

Antigen-derived peptides are tethered within the binding groove of the class II MHC heterodimer by an elaborate hydrogen-bond network involving main-chain amide protons and carbonyl oxygen atoms, together with interactions of several constituent amino acid side chains within individual MHC pockets (Stern *et al.*, 1994). It is the extensive allelic polymorphism within these latter subsites that results in each αβ dimer exhibiting the capacity to bind a distinct repertoire of peptides (Hurley & Steiner, 1995). This study has revealed that CLIP sustains an interaction with different allelic variants of the I-A isotype, $I\text{-}A^a$, $I\text{-}A^d$ and $I\text{-}A^k$, not by forming strong side-chain interactions (*Chapter 3*) but, rather, through a network of main-chain hydrogen bonds (*Chapter 4*). The phylogenetically-conserved central continuous region, CLIP91–99, is intolerant of backbone stereochemical disruptions by serial D-alanine-substitutions. Experiments with truncated and frame-shift analogues of CLIP showed that for effective binding to I-A molecules, the sequence element CLIP90–100 must be incorporated into a peptide of thirteen or more residues including at least three residues N-terminal to this motif.

A measure of positive side-chain interaction with I-A molecules occurred *via* Thr 95, Leu 98 and Met 102, which were predicted to contact the class II MHC heterodimer outside of the immediate environment of the polymorphic pockets of the peptide-binding groove (*Chapter 3*). Similarly, the binding of CLIP to a representative mouse class II MHC variant of the I-E isotype involved the formation of an elaborate hydrogen bonding scheme *via* the main-chain atoms of the peptide, while anchoring contacts were apparent between the same CLIP side chains and the $I\text{-}E^d$ αβ dimer at regions of the molecule distinct from the pockets of the groove (*Chapter 5*).

The relationship between the structure and binding of CLIP analogues suggests that there is a general backbone motif of a periodic nature within this sequence that minimises deleterious contacts and allows promiscuous association with class II MHC molecules. However, several conserved inhibitory contacts modulate this affinity such that CLIP may dissociate enabling efficient presentation of antigen-derived peptides.

The binding of CLIP to I-A^{g7} from non-obese diabetic (NOD) mice has also been shown to be dependent upon a number of critical anchor contacts made through the C-terminal residues of the peptide sequence in addition to the main-chain hydrogen bond network observed in other CLIP–I-A complexes (*Chapter 6*). Moreover, the βAsp57Ser mutation in I-A^{g7} confers a strong preference for peptides that exhibit an acidic residue at the P9 position, while the βPro56His mutation appears to reduce the extent of ligand exchange that takes place at the cell surface. The possible ways in which these properties of I-A^{g7} may contribute to the development in autoimmunity in NOD mice are discussed.

PUBLICATIONS

Weenink, S. M. and Gautam, A. M. (1997) Antigen presentation by MHC class II molecules. *Immunol. Cell Biol.* **75:** 69–81. *(a review)*

Weenink, S. M., Milburn, P. J. and Gautam, A. M. (1997) A continuous central motif of invariant chain peptides, CLIP, is essential for binding to various I-A MHC class II molecules. *Int. Immunol.* **9:** 317–325.

Weenink, S. M., Averdunk, H., Boston, T., Boswarva, V., Guery, J.-C., Adorini, L., Mellins, E., McCluskey, J. and Gautam, A. M. (1997) Impaired antigen presentation by murine I-A^d class II MHC molecules expressed in normal and HLA-DM-defective human B cell lines. *Int. Immunol.* **9:** 889–896.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

ABTS	2, 2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
Ag	antigen
APC	antigen-presenting cell
ATCC	American type culture collection
ATP	adenosine 5'-triphosphate
BCG	bacillus Calmette-Guérin
BCR	B cell receptor
bp	base pair
BSA	bovine serum albumin
°C	degrees Celsius
CD	circular dichroism/ cluster of differentiation cell-surface marker (*e.g.* CD4)
cDNA	complementary DNA
CFA	complete Freund's adjuvant
CIITA	class II MHC transactivator protein
CIIV	class II MHC-containing vesicle
CLIP	class II MHC-associated invariant chain peptides
ddH_2O	deionised distilled water
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
EAE	experimental allergic encephalomyelitis
EBV	Epstein-Barr virus
ECL	enhanced chemiluminescence
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EMS	ethyl methane sulphonate
ER	endoplasmic reticulum
EtOH	ethanol
FACS	fluorescence-activated cell sorter
FBS	fœtal bovine serum
FITC	fluorescein isothiocyante
g	relative gravitational unit
GAD	glutamic acid decarboxylase
h	hour
H-2	mouse major histocompatibility complex
HA	hæmagglutinin
HEL	hen egg lysozyme
HEPES	*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid
HLA	human leucocyte antigen, human major histocompatibility complex

HPLC	high performance liquid chromatography
I-A	mouse class II MHC heterodimer encoded by *Aa* and *Ab* genes
IC_{50}	concentration of competitor which yields 50% inhibition
IDDM	insulin-dependent diabetes mellitus
I-E	mouse class II MHC heterodimer encoded by *Ea* and *Eb/Eb2* genes
IFN	interferon (*e.g.* IFN-γ)
Ig	immunoglobulin (*e.g.* IgG, IgM)
IGEPAL	*tert*-octylphenoxy poly(oxyethylene) ethanol
Ii	invariant chain
IL	interleukin (*e.g.* IL-2)
kDa	kiloDalton
L	litre
LAMP	lysosomal-associated membrane protein (*e.g.* LAMP-1)
LCL	lymphoblastoid cell line
LIP	leupeptin-induced polypeptides
mAb	monoclonal antibody
MBP	myelin basic protein
mCyt*c*	moth cytochrome *c*
2-ME	2-mercaptoethanol
MeOH	methanol
MFI	median fluorescence intensity
mg	milligram
MHC	major histocompatibility complex
MIIC	MHC class II compartment
min	minute
mL	millilitres
MMTV	mouse mammary tumour virus
MPR	mannose-6-phosphate receptor
M_r	relative molecular mass
MRA	mycoplasma removal agent
MS	multiple sclerosis
MSA	mouse serum albumin
MW	molecular weight
NOD	non-obese diabetic mouse
nm	nanometre
NP-40	nonidet P-40, polyoxyethylene (9) *p-t*-octyl phenol
OD	optical density
Ova	chicken egg ovalbumin
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PI	propidium iodide
PLP	proteolipid protein
PMSF	phenylmethylsulphonyl fluoride

rMOG	rat myelin oligodendrocyte glycoprotein
rpm	revolutions *per* minute
RPMI	Roswell Park Memorial Institute tissue culture medium
s	second
SAg	superantigen
SDS	sodium dodecyl sulphate
SE	*Staphylococcus aureus* enterotoxin (*e.g.* SEA)
SLIP	small leupeptin-induced peptides
SWM	sperm whale myoglobin
TAP	transporter associated with antigen processing
TCR	T cell receptor
TGN	*trans*-Golgi network
TFA	trifluoroacetic acid
TfR	transferrin receptor
Tricine	*N*-((trishydroxymethyl)methyl)glycine
Tris	tris(hydroxymethyl) aminomethane
TSST-1	toxic shock syndrome toxin-1
Tween-20	polyoxyethylene (20) sorbitol monolaurate
UV	ultraviolet
V	volts
v/v	volume for volume
w/v	weight for volume
w/w	weight for weight

CHAPTER 1.

General introduction

1.1. The immune system and the major histocompatibility complex (MHC)

In higher vertebrates, many proteins implicated in immunological processes are encoded within a single genetic region known as the major histocompatibility complex (MHC). In humans, the MHC spans nearly 4000 kb in the short arm of chromosome 6 and its gene products are referred to as HLA (human leucocyte antigens; Figure 1.1a). The mouse MHC genetic region is known as the H-2 complex and has been mapped to chromosome 17 (Figure 1.1b).

A critical requirement of the immune system is the ability to differentiate self from foreign (non-self) matter and eliminate the latter. The significance of MHC-encoded molecules in the adaptive immune response was first recognised from their ability to provoke vigorous rejection of intra-species tissue grafts (Gorer, 1936, 1937; Snell, 1948). Of particular interest in this reaction are the class I and class II MHC molecules. These membrane-bound glycoprotein receptors bind fragments of protein antigen and display them at the cell surface. An immune response is initiated when the peptide ligand is recognised and engaged by the highly specific receptors of T cells (TCR) in the context of the MHC molecule. The dual requirement for a T cell to be able to recognise not only the peptide antigen but also the host MHC molecule in the trimolecular complex is called MHC restriction (Zinkernagel & Doherty, 1974).

1.2. A division of labour: class I and class II MHC molecules

Together, class I and class II MHC molecules enable the vertebrate immune system to maintain complete surveillance of the cellular environment for any evidence of foreign metabolic activity. The most significant distinction between these two populations of MHC molecules is a functional one based upon their site of ligand loading.

Class I MHC molecules are expressed by almost all nucleated cells and, typically, bind peptides in the endoplasmic reticulum (ER) that are derived from protein antigen residing in the cytosol. These proteins may include those generated by intracellular pathogens, such as viruses or parasites. The proteolytic machinery of the proteasome complex degrades these antigens into short peptide fragments which are carried into the lumen of the ER by TAP (transporter associated with antigen processing), an ATP-binding heterodimer comprising the MHC-encoded gene products, TAP-1 and TAP-2. Within

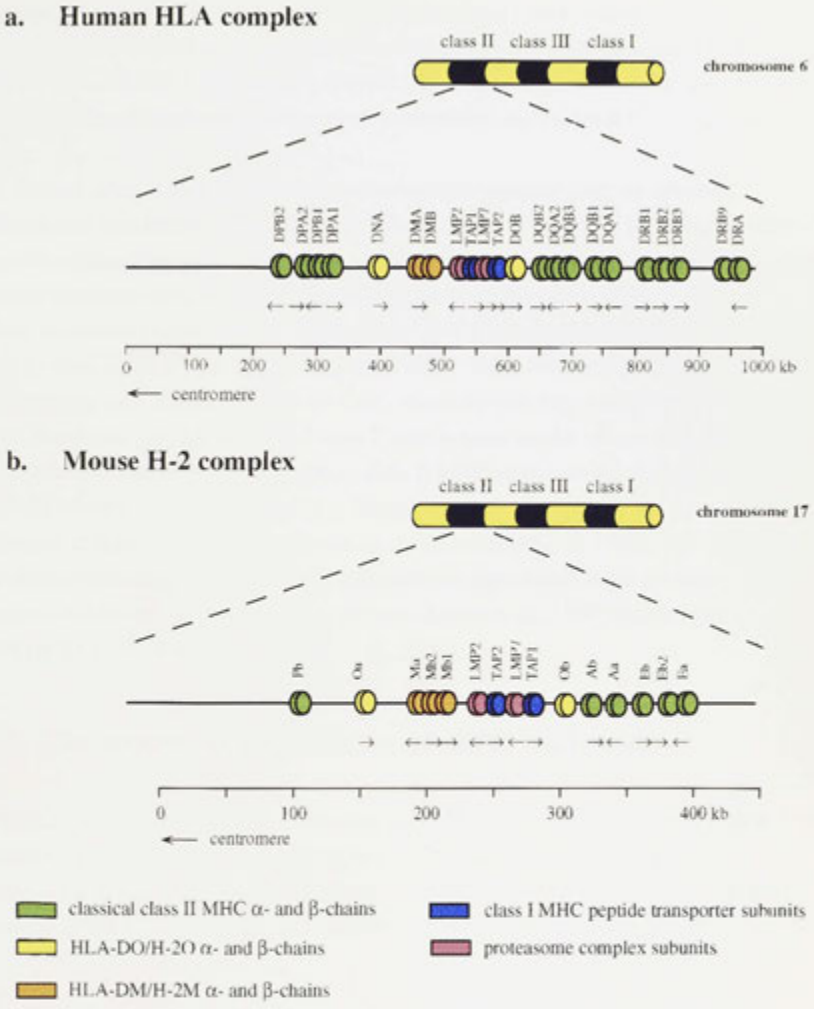


Figure 1.1 *The class II region of the major histocompatibility complex.*

The genetic region known as the major histocompatibility complex (MHC) consists of three classes of genes. Class I and II MHC gene products are involved in presentation of peptide antigens to T cells. The class III region encodes a mixture of proteins, only some involved in immunological processes. The human class II MHC region **(a)** spans 1000 kb region on the short arm of chromosome 6. The genes encoding the classical class II MHC molecules are clustered in three major polymorphic subregions, *HLA-DR, -DP,* and *-DQ.* Not all known loci localised within this region are shown. The mouse class II MHC region **(b)** has been mapped to the short arm of chromosome 17. Subunits of classical mouse class II MHC molecules are encoded by *H2-A* and *-E* loci. The *DRA* and *Ea* genes are invariant (Lee *et al.*, 1982; Das *et al.*, 1983; Ayane *et al.*, 1986). The gene products of other loci found within the class II MHC region are indicated in the legend. Horizontal arrows indicate direction of transcription.

this site, the peptides bind to the newly-synthesised class I MHC molecules and the now-stable complexes travel *via* the constitutive secretory pathway to the cell surface for presentation to $CD8^+$ T lymphocytes (Figure 1.2). For a comprehensive review of the class I MHC-mediated pathway of antigen presentation, see Pamer & Cresswell (1998).

By contrast, class II MHC molecules present peptides sampled from the subset of proteins that gain access to the endocytic compartments, for example, by endocytosis at the cell surface (Figure 1.2). Thus, the peptide repertoire of class II MHC molecules may include fragments derived from proteins of extracellular pathogens, such as bacteria. These molecules present their peptide cargo to $CD4^+$ T lymphocytes and are expressed only by specialised antigen-presenting cells (APC). These APC include B lymphocytes, macrophages (*e.g.* Kupffer cells in the liver), dendritic cells (*e.g.* Langerhans cells of the skin), the thymic epithelium and activated T cells in most species except the mouse. Other cell types may also be induced to express class II MHC molecules through the actions of external stimuli, such as cytokines (*e.g.* Wright *et al.*, 1986; Pujol-Borrell *et al.*, 1987; Glimcher & Kara, 1992; Bourdoulous *et al.*, 1993; Albanesi *et al.*, 1998). Alterations in the tissue distribution of class II MHC molecules has been linked to the development of a number of diseases, including Crohn's disease (Koretz *et al.*, 1987; Momburg *et al.*, 1988) and Grave's disease (Catálfamo *et al.*, 1999).

1.3. The structural organisation of MHC molecules

Research over the last decade has advanced our understanding of antigen presentation considerably, in particular, how peptide ligands interact with MHC molecules. In molecular terms, a major breakthrough was the elucidation of the X-ray crystallographic structure of the human class I MHC molecule, HLA-A2 (Bjorkman *et al.*, 1987b).

1.3.1 Class I MHC molecules

The product of the class I MHC-encoded gene is a 44–45 kDa transmembrane glycoprotein that possesses a short carboxy-terminal cytoplasmic domain (~30 amino acids), a single transmembrane domain (~25 amino acids) and three NH_2-terminal extracellular domains (α_1, α_2, α_3) each of 90–92 amino acids (reviewed by Brodsky & Guagliardi, 1991). A peptide-receptive class I MHC molecule is created when this heavy chain forms a non-covalent association with the second subunit, the 12 kDa, non-MHC-encoded, soluble light chain, β_2-microglobulin (β_2m).

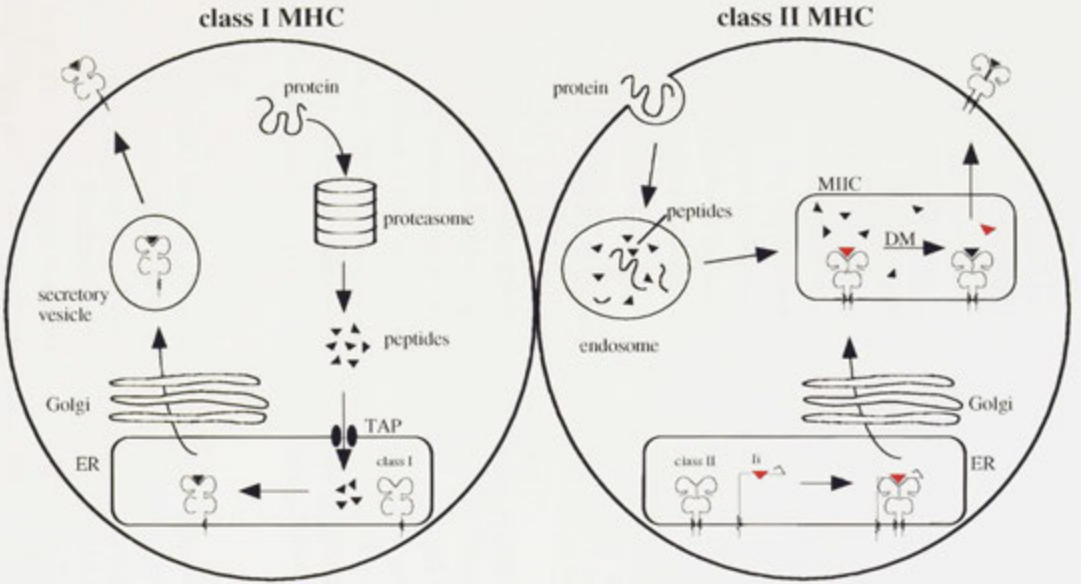


Figure 1.2 *Schematic outline of the class I and class II MHC antigen processing and presentation pathways.*

The two pathways of MHC-mediated antigen presentation enable the vertebrate immune system to maintain surveillance of both the extracellular and intracellular environments by presenting fragments of foreign proteins, as well as many self-proteins, to the circulating T-cell population. The class I MHC subunits, the heavy chain and β_2m associate together in the endoplasmic reticulum (ER) chaperoned by calnexin (details not shown) then bind peptides delivered into the ER by the TAP complex. The binding of peptide stabilises the class I MHC molecule and it moves *via* the secretory pathway to the cell surface. In the class II MHC pathway of antigen presentation, the α and β subunits associate in the ER followed by the interaction of a third polypeptide, the invariant chain (Ii). Nonameric $\alpha\beta$Ii complexes traverse the Golgi compartment into the endocytic route. In the MIIC (MHC class II compartments), the invariant chain is degraded progressively and dissociates from each $\alpha\beta$ dimer leaving CLIP (class II MHC-associated invariant chain peptides; shown in red). HLA-DM facilitates the dissociation of CLIP for the loading of peptides derived from exogenous protein antigens that have been endocytosed at the cell surface.

The three-dimensional analysis of HLA-A2 revealed that the class I MHC molecule exhibits two distinct extracellular structural motifs (Figure 1.3; Bjorkman *et al.*, 1987b). At the membrane-proximal end, the α_3 domain of the heavy chain and the β_2m form an immunoglobulin-type structure. Most significantly, at the membrane-distal end, the α_1 and α_2 domains of the heavy chain form a deep cleft comprising a platform of a single eight-stranded, anti-parallel β-pleated sheet, topped by two anti-parallel α-helices. This represents the site at which the peptide antigen binds (Bjorkman *et al.*, 1987b).

The class I MHC peptide-binding groove is effectively closed at each end by the α-helix of the α_1 domain. The dimensions of this space permit a peptide of 8–11 amino acids to bind in an extended conformation (Madden *et al.*, 1991). Peptides at the larger end of this length range may exhibit a kink in the centre in order for their terminal residues to be accommodated. The extended conformation of the peptide results in the side chains of the constituent amino acids extending from the groove in a roughly alternating pattern. It is this composite surface of peptide bound within the MHC groove that is recognised by the TCR in the trimolecular complex (Bjorkman *et al.*, 1987a).

1.3.2 Class II MHC molecules

Owing to the overall similarities in both sequence and the location of conserved and polymorphic residues between class I and class II MHC molecules, a model of the class II MHC protein was proposed in 1988 based upon the molecular details of the class I MHC structure (Brown *et al.*, 1988). A number of class II MHC molecules of both human (Brown *et al.*, 1993; Jardetzky *et al.*, 1994; Stern *et al.*, 1994; Ghosh *et al.*, 1995; Dessen *et al.*, 1997) and mouse (Fremont *et al.*, 1996, 1998b; Scott *et al.*, 1998) origins have now been analysed by X-ray crystallography and have confirmed the main features of this model.

Like class I MHC molecules, those of the class II MHC consist of a non-covalently-linked heterodimer. In this case, both glycoprotein chains are encoded within the MHC genetic region and span the membrane bilayer (Figure 1.3; reviewed by Brodsky & Guagliardi, 1991). The class II MHC α-subunit has a relative molecular mass (M_r) of 33–35 kDa and carries two N-linked oligosaccharides. The M_r of the β-subunit is 25–30 kDa and this polypeptide is modified by a single N-linked carbohydrate moiety. Similar to the class I MHC heavy chain, each class II MHC subunit exhibits two extracellular N-terminal domains of 90–100 amino acids, a single transmembrane domain of 20–25 amino acids and a short carboxyl-terminal cytoplasmic domain of 10–15 amino acids.

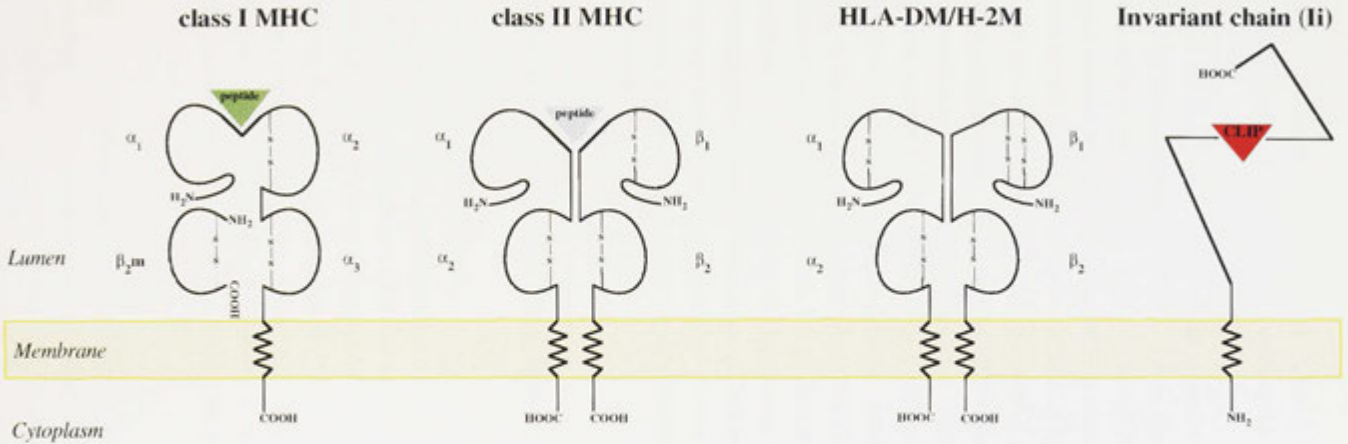


Figure 1.3 *Domain organisation of the molecular components of MHC-mediated antigen presentation.*

MHC antigen presentation is mediated by two different glycoprotein heterodimers. Class I MHC molecules consist of a large 44–45 kDa transmembrane polypeptide with three extracellular domains (α_1, α_2 & α_3), non-covalently associated with the smaller 12 kDa β_2-microglobulin (β_2m). Class II MHC molecules are composed of two integral membrane polypeptide chains, the α-chain (33–35 kDa) and β-chain (25–30 kDa), each folding to yield two lumenal domains. Antigen-derived peptides bind to both classes of MHC molecule within a cleft formed at the protein surface by the membrane-distal domains. Like the class II MHC molecule, HLA-DM (H-2M in the mouse) consists of an αβ heterodimer but its groove is unable to accomodate a peptide due to close packing of the α_1 and β_1 domains and additional disulphide bonds. The invariant chain (Ii) is a type II integral membrane protein, inserted into the membrane in an inverted polarity relative to other transmembrane proteins. The primary interaction site between Ii and class II MHC molecules is the exon 3-derived CLIP region (shown in red). See text for references.

The membrane-distal domains of each subunit (α_1 and β_1) associate to form a structure that constitutes the peptide-binding region of the molecule.

The structure of the class II MHC peptide-binding groove resembles that of class I MHC molecules except for two significant features. Firstly, the two polypeptide components of the heterodimer, the α- and β-chains, contribute equally to its formation, with four of the eight β-strands and one helical region being derived from each subunit (Brown *et al.*, 1993). Secondly, two turns near the amino terminus of the class I MHC α_1 helical region are replaced in the class II MHC structure by a stretch of extended chain. In addition, the specific amino acid side chains that occlude the ends of the class I MHC peptide-binding site are not present in the class II MHC heterodimer. These latter structural features leave the class II MHC peptide-binding groove open at both ends, thereby allowing the NH_2- and COOH- termini of a bound peptide to protrude. Accordingly, ligands eluted from class II MHC molecules are typically longer than class I MHC peptides, measuring 12–19 amino residues in length (Rudensky *et al.*, 1991; Chicz *et al.*, 1992, 1993; Hunt *et al.*, 1992). Within the class II MHC peptide-binding groove, these ligands are bound in a straight, extended conformation with a pronounced left-handed twist, such that successive side chains project from the backbone approximately every -130° (Stern *et al.*, 1994). This arrangement is similar to a type II polyproline helix, which has an angle of projection of -120° *per* residue.

1.3.3 Interactions at the peptide–MHC molecule interface

Two types of intermolecular interactions provide positive binding energy to secure a peptide ligand within the MHC peptide-binding groove. The first of these is the formation of hydrogen bonds between main-chain atoms of the peptide backbone and highly-conserved residues of the MHC heterodimer. Within class I MHC molecules, such residues are clustered at each end of the cleft, situated to contact the first and last residues of the peptide (Madden *et al.*, 1991). Within the open-ended groove of class II MHC molecules, conserved residues with hydrogen-bonding potential are distributed more evenly and these bonds form at intervals along the peptide backbone (Brown *et al.*, 1993; Figure 1.4a).

The formation of main-chain hydrogen bonds between the MHC peptide-binding groove and the ligand represents a source of positive binding energy that is entirely independent of the peptide sequence. This is not the case with the second type of interaction between peptide ligand and MHC receptor. The primary sequences of both class I and class II

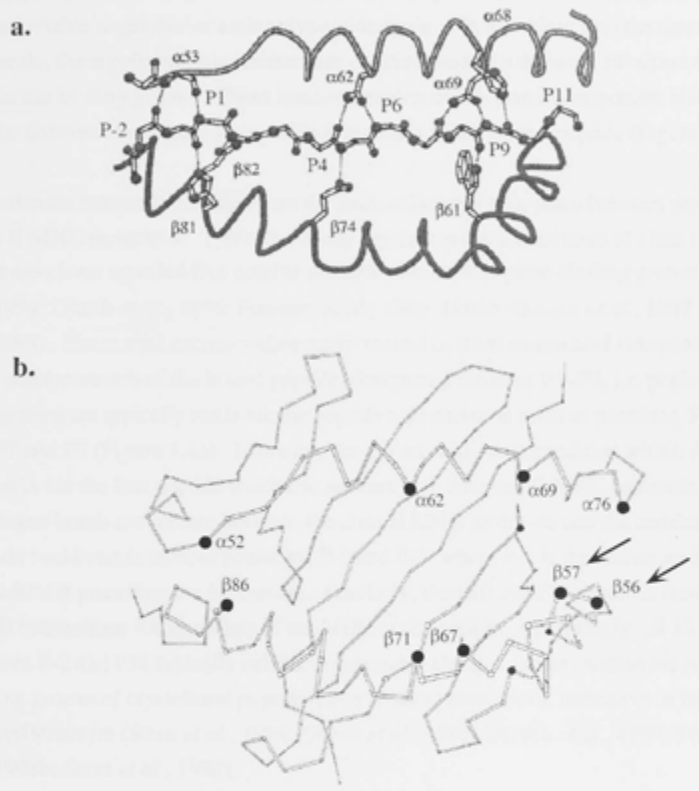


Figure 1.4 *The class II MHC peptide-binding groove*

Antigen-derived peptides are tethered within the cleft of class II MHC molecules by interactions involving the side chains of the individual residues and/or the main-chain atoms of the polyamide backbone. **(a)** The peptide-binding groove typically encompasses a nine-residue stretch of the bound peptide (P1–P9). An elaborate network of hydrogen bonds forms between peptide main-chain carbonyl oxygen atoms and amide protons and highly-conserved residues of the MHC heterodimer. Shown is the hydrogen-bonding scheme between the ovalbumin peptide, Ova323–339, and the mouse class II MHC molecule, I-A^d (adapted from Scott *et al.*, 1998). Five notable pockets exist within the class II MHC peptide-binding groove. These sites accommodate every second or third amino acid side chain of the bound ligand. Pocket interactions are typically made *via* the peptide side chains at relative positions, P1, P4, P6, P7 and P9. The intervening peptide residues, P2, P3, P5 and P8 are oriented sideways across the groove or project outwards from this site to establish TCR contact points. The amino acid composition of each pocket varies considerably between individual MHC allotypes which results in different preferential peptide-sequence binding motifs. Amino acid positions of particular interest are shown in **(b)** on the α-carbon backbone trace of the human class II MHC molecule, HLA-DR1 (adapted from Hurley & Steiner, 1995). The arrows highlight the residues at positions β56 and β57 which are of significance in the I-A^{g7} class II MHC molecule of non-obese diabetic (NOD) mice (discussed in *Chapter 6*). Throughout this thesis, I-A class II MHC residues are numbered according to the secondary structure-based scheme proposed by Fremont *et al.* (1998b) in order to be consistent with previous alignments of I-E and HLA-DR (Kabat, 1991). The nomenclature used to define the individual regions of secondary structure within the groove is also given in Fremont *et al.* (1998b).

MHC gene products exhibit extreme allelic diversity within defined subregions or 'hypervariable segments' of each polypeptide chain. Upon folding into the mature MHC molecule, these polymorphic residues are concentrated into different subsites or 'pockets' within the binding groove. These localised environments provide important binding sites for the downward-oriented amino acid side chains of the bound peptide (Figure 1.4b).

Of particular interest in this study are the interactions that take place between peptides and class II MHC molecules. Typically, X-ray crystallographic structures of class II MHC αβ dimers have revealed five notable pockets within the peptide-binding groove (Stern *et al.*, 1994; Ghosh *et al.*, 1995; Fremont *et al.*, 1996, 1998b; Dessen *et al.*, 1997; Scott *et al.*, 1998). These sites accommodate every second or third amino acid side chain within a nine-residue stretch of the bound peptide, designated residues P1–P9, *i.e.* pocket interactions are typically made *via* the peptide side chains at relative positions, P1, P4, P6, P7 and P9 (Figure 1.4a). However, the amino acid accommodated within the P1 pocket is not the first peptide residue to interact with the class II MHC molecule. Hydrogen bonds are present between the class II MHC molecule and the residues of the peptide backbone at relative positions, P-1 and P-2, where P-1 is the amino acid immediately preceding the P1 residue. Similarly, the P10 and P11 residues form van der Waals interactions with residues of the MHC binding cleft. Accordingly, all 13 residues between P-2 and P11 typically exhibit interpretable electron density within the peptide-binding groove of crystallised peptide–class II MHC complexes, indicative of having ordered structure (Stern *et al.*, 1994; Ghosh *et al.*, 1995; Dessen *et al.*, 1997; Fremont *et al.*, 1998b; Scott *et al.*, 1998).

The distinct amino acid composition of each pocket within the binding groove of a given MHC molecule results in these sites displaying preferences for peptide residues of particular size and physicochemical characteristics. The binding of such residues provides positive binding energy to the complex, for example, though the fulfilment of electrostatic or hydrophobic requirements. The most important of these preferences are referred to as 'anchor' positions within the peptide sequence. For the purposes of this study, an anchor residue shall be defined as one that provides positive binding energy to stabilise the resultant complex to the extent that it will not form in its absence. Thus, MHC molecules that differ in their primary sequence will exhibit different anchor preferences and, similarly, distinct peptide-binding sequence motifs. This represents the means by which different MHC allelic variants may bind diverse sets of peptides (Hurley & Steiner, 1995; Rammensee *et al.*, 1995).

The residues within a peptide sequence do not act independently of each other but rather may be influenced by interactions at neighbouring positions. For example, favourable contacts between several peptide side chains within just a subset of the available pockets may overcome inhibitory interactions at adjacent sites. In this way, each MHC molecule may accommodate a huge array of different peptides by various combinations of optimally- and suboptimally-occupied pockets. It has been estimated that between 650–2000 structurally-divergent peptide sequences may bind to the single mouse class II MHC variant, I-A^d (Hunt *et al.*, 1992).

The diversity of peptides presented by both a single and a set of distinct MHC allotypes is of considerable functional significance. The entire pool of MHC alleles within a population is extensive — more than 50 at some loci. However, each individual may express only between 3–6 of these variants depending on whether they are inherited in a homozygous or heterozygous state. Together, these MHC molecules must exhibit great versatility in the range of peptides they may bind in order to ensure adequate presentation of the proteolysed remains of each and every foreign invader. The efficiency of this process is reflected in the variation observed between individuals with respect to susceptibility to different diseases. For some pathological conditions, the HLA alleles expressed may be the primary risk factor (Thorsby, 1995).

1.3.4 The nomenclature of class I and class II MHC molecules

An examination of the different molecules encoded within the MHC requires first an understanding of both the terminology used to describe them and the nomenclature applied to classify them. In both humans and mice, three distinct forms or 'isotypes' of the class I MHC molecule are expressed, HLA-A, HLA-B, HLA-C and H-2K, H-2D, H-2L, respectively. Humans also express three class II MHC isotypes, HLA-DR, HLA-DP and HLA-DQ, but only two are present in mice, H-2A and H-2E. These are homologous to HLA-DQ and HLA-DR, respectively. The mouse gene equivalent to that encoding the HLA-DP β-chain, *Pb* (formerly *Aβ3*), is an untranscribed pseudogene (Widera & Flavell, 1985) and no corresponding mouse DP α-chain gene has been found (Figure 1.1).

Within the genetic locus of each class II MHC isotype, there exist a number of subregions that encode different forms of the α- and β-chain polypeptides of the protein heterodimer, *e.g. HLA-DQA1*. The gene products of these sub-loci may be classified further between different individuals into allelic variants or 'allotypes'. Such tissue typing of HLA gene products was conducted originally by serological means through the assessment of

different antibody reactivities. More recently, the development of molecular biology techniques, such as restriction fragment length polymorphism (RFLP) analysis and the polymerase chain reaction (PCR), has allowed HLA polymorphism to be characterised more precisely at the level of the DNA sequence (reviewed by Corzo *et al.*, 1995 and Wade, 1996). For example, the serologically-defined HLA-DQ8 αβ dimer may be represented more accurately using the nomenclature which defines its allelic composition, HLA-DQA1*0302/HLA-DQB1*0302.

The set of alleles within the MHC of a particular individual is called the haplotype. Such terminology is particularly important in the description of the MHC molecules expressed by different inbred strains of mice. Each strain has been assigned an arbitrary haplotype code written in superscript. For example, the collection of MHC alleles expressed by mice of the BALB/c strain has been designated the *d* haplotype or H-2^d (Coligan *et al.*, 1994). Accordingly, the individual alleles expressed by these animals are named *H-2K^d*, *H-2D^d*, *H-2Aa^d*, *H-2Eb^d* and so on. The gene products are named similarly, for example, H-2K^d, H-2D^d, H-2A^d and H-2E^d. The mouse class II MHC molecules are also known more simply as I-E^d and I-A^d, using the nomenclature derived from the original description of these molecules as the immune response-associated (Ia) antigens encoded by the immune response (*Ir*) genes within the *I* region of the mouse MHC (Benacerraf, 1981; Klein *et al.*, 1981).

Lastly, it should be noted that the α-chain sequences of HLA-DR and H-2E class II MHC molecules exhibit minimal polymorphism between individuals (*e.g.* Korman *et al.*, 1982; Larhammar *et al.*, 1982; Lee *et al.*, 1982; Das *et al.*, 1983; Mengle-Gaw & McDevitt, 1985; Ayane *et al.*, 1986). The alleles encoding these polypeptides may be written simply as *HLA-DRA* and *H-2Ea*, respectively.

1.3.5 Conformational features of peptide-bound class II MHC molecules

Aside from the obvious functional necessity, both class I and class II MHC molecules must bind peptide ligand for structural maturation. The association of peptide provides critical stabilising interactions that bring about conformational rearrangements in the molecular architecture to yield molecules stable enough to withstand the rigours of antigen presentation at the cell surface. Without it, these molecules are folded poorly, exhibit aberrant glycosylation and are likely to accumulate intracellularly in aggregates in the ER and be targeted for degradation (Townsend *et al.*, 1989; Ljunggren *et al.*, 1990; Germain & Rinker, 1993).

The conformational states of both class I and II MHC molecules may be distinguished by their reactivity with different antibodies (Reske & Weitzel, 1985; Mellins *et al.*, 1990; Elliott *et al.*, 1991; Germain & Hendrix, 1991). Additionally, class II MHC molecules may be analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) to provide a convenient, if artificial, means of assessing conformational features. Upon SDS-PAGE, without prior heat denaturation and in the absence of reducing agents, class II MHC molecules show 3 distinct forms (Sadegh-Nasseri & Germain, 1991). The so-called 'compact' or C dimers migrate with an approximate molecular weight of 63 kDa in human (52–56 kDa in mouse) and are formed by most (Germain & Hendrix, 1991) but not all peptide-bound class II MHC αβ dimers (*e.g.* Mellins *et al.*, 1990; Chicz *et al.*, 1992; Riberdy *et al.*, 1992). The 'floppy' or F dimers migrate in a diffuse band at 63–67 kDa and represent an intermediate, partially-dissociated form that occurs under mild denaturation conditions. Finally, in the complete absence of any stabilising influence of bound peptide, the class II MHC molecule is easily denatured in the presence of SDS detergent and migrates as disassembled α- and β-monomers.

1.4. The interaction of class II MHC molecules with the invariant chain, Ii

For the efficient functioning of the class II MHC pathway of antigen presentation, it is essential that these αβ dimers do not acquire antigen-derived peptides within the ER. Yet at the same time, the unstable, empty molecules must remain in a peptide-receptive state, avoid aggregation and be transported into the endocytic route. All of these events are mediated through the interaction with a third, non-polymorphic polypeptide, the invariant chain (Ii).

1.4.1 The structure of the invariant chain protein

Ii was first identified by two-dimensional PAGE of immunoprecipitates of mouse I-A and I-E class II MHC molecules (Jones *et al.*, 1978a). The protein exhibits an unusual amino acid composition, being particularly rich in methionine residues and also contains large numbers of the basic residues, lysine and arginine, to give it a pI value between 8.0–8.5 (McMillan *et al.*, 1981). An examination of the deduced amino acid sequence of the human (*Homo sapiens*; Strubin *et al.*, 1984, 1986a; Kudo *et al.*, 1985) mouse (*Mus musculus*; Koch *et al.*, 1987), rat (*Rattus norvegicus*; McKnight *et al.*, 1989) and cow Ii

protein (*Bos taurus*; Niimi *et al.*, 1996) reveals minimal polymorphism between the different species (Figure 1.5). For example, human and mouse Ii exhibit 76% sequence identity at the protein level.

Ii is a type II, integral membrane protein, meaning that it lacks a cleavable signal sequence and is therefore oriented in the membrane in an inverted polarity compared with most transmembrane proteins, including the α- and β-chains of the class II MHC heterodimer, *i.e.* with its carboxy-terminus on the lumenal side and its amino-terminus exposed to the cytoplasm (Singer *et al.*, 1984; Strubin *et al.*, 1984; Lipp & Dobberstein, 1986; Figure 1.3). The Ii gene is not linked to the MHC (Day & Jones, 1983) but, rather, is encoded chromosome 18 in mice (Richards *et al.*, 1985; Yamamoto *et al.*, 1985) and on the long arm of chromosome 5, band q32, in humans (Claesson-Welsh *et al.*, 1984; Genuardi & Saunders, 1988). However, the expression of this gene is regulated by the same mechanisms that control the expression of the class II MHC subunits, for example, through the binding of the class II MHC transactivator protein (CIITA) to the promoter region (Chang & Flavell, 1995).

The Ii protein exhibits a short amino-terminal cytoplasmic tail, a single transmembrane domain (approximately 26 amino acids) and a large, lumenal carboxyl-terminal domain (Claesson *et al.*, 1983; Figure 1.3). Human Ii exits in 4 isoforms of relative molecular mass, 31–33 kDa, 33–35 kDa, 41–43 kDa and 43–45 kDa (Strubin *et al.*, 1986a; O'Sullivan *et al.*, 1987; Figure 1.6). The range in M_r observed for each form reflects minor variations in their rate of migration upon SDS-PAGE. Within this thesis, these forms shall be referred to hereafter as p31, p33, p41 and p43, respectively. The p31 and p41 Ii proteins are derived by the alternate splicing of an additional exon, 6b, which extends the extracellular carboxyl-terminal domain by 64 amino acids to add a cysteine-rich, thyroglobulin type 1-like domain (Strubin *et al.*, 1986a; O'Sullivan *et al.*, 1987). The p33 and p43 forms arise as a result of a second in-phase AUG codon for the initiation of translation and thus represent N-terminally-extended forms of p31 and p41, respectively (Strubin *et al.*, 1986b; O'Sullivan *et al.*, 1987). The mouse, rat and bovine Ii genes do not appear to exhibit the alternative initiation codon upstream and as a result these species do not synthesise the p33 or p43 Ii variants (Koch *et al.*, 1987; McKnight *et al.*, 1989; Niimi *et al.*, 1996). In human B cells, the most abundant form is p31, for which there is a 10-fold excess of mRNA over that encoding p41 (Strubin *et al.*, 1986a).

Throughout this thesis, Ii and all Ii-derived peptides, both mouse and human, are numbered according to the Ii sequence alignment shown in Figure 1.5. The mature Ii protein displays extensive co-translational and post-translational modifications, including

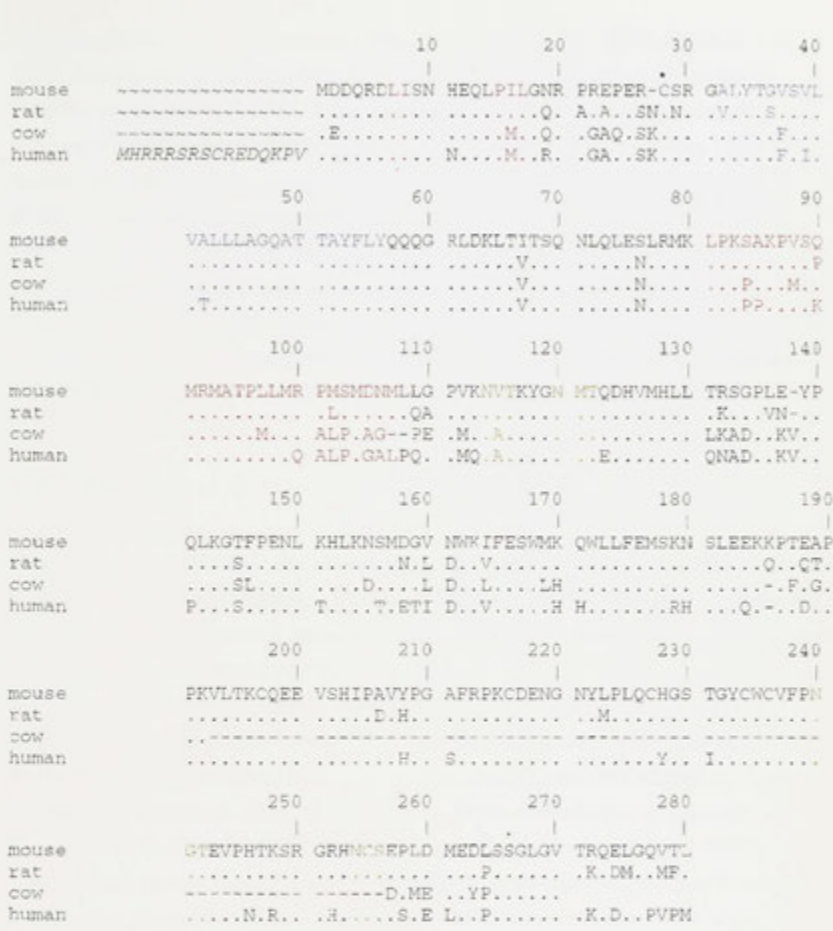


Figure 1.5 *Sequence alignment of the invariant chain protein.*

The amino acid sequence of the mouse invariant chain protein, deduced from cDNA clones (*Mus musculus*; Koch *et al.*, 1987), is aligned with rat (*Rattus norvegicus*; McKnight *et al.*, 1989), cow (*Bos taurus*; Niimi *et al.*, 1996) and human Ii (*Homo sapiens*; Strubin *et al.*, 1984, 1986a; Kudo *et al.*, 1985). Sequences are given in single letter amino acid code and numbered with reference to human Ii. The mouse Ii protein exhibits deletions at positions 27 and 138 plus an insertion at 186 relative to human p43. Putative *N*-linked glycosylation sites are marked in green. The stretch of amino acids of the transmembrane region are coloured blue. The 16 amino acids of the N-terminal extension of human p43 are in italics. Putative sorting signals for endosomal compartments are indicated by magenta shading. The exon 3-derived CLIP segment is coloured red. The additional 64 amino acid element encoded by the 6b exon is highly homologous to a thyroglobulin repeat (TgR). This was not seen in the sequenced bovine Ii clone and is represented by dashes. The cysteine residue to which palmitic acid is bound (Cys 28) is marked with an asterisk. The serine residue to which chondroitin sulphate is conjugated (Ser 202 in p31 Ii) is marked with a dot.

phosphorylation (Spiro & Quaranta, 1989; Anderson & Roche, 1998; Kuwana *et al.*, 1998; Anderson *et al.*, 1999b), both N- and O-linked glycosylation and sialylation (Machamer & Cresswell, 1982, 1984; Schröder *et al.*, 1999) and the addition of a palmitic acid chain *via* a thioester linkage to Cys 28 (Koch & Hämmerling, 1985, 1986; Simonis & Cullen, 1986). A small proportion of Ii (2–5%) is also modified to a proteoglycan by the addition of a chondroitin sulphate moiety to Ser 202 (Sant *et al.*, 1985; Giacoletto *et al.*, 1986; Miller *et al.*, 1988). Although Ii does not represent a typical structural component of class II MHC antigens at the cell surface (Sung & Jones, 1981), these molecules may be the exception. This modified form of Ii is believed to act like an accessory molecule, enhancing the stimulation of T cell responses through interaction with CD44 (Naujokas *et al.*, 1993). Cell-surface Ii has been designated CD74.

1.4.2 The roles of Ii

Shortly after biosynthesis in the ER, the α- and β-subunits of the class II MHC molecules associate to form non-covalently-linked heterodimers (Figure 1.2). Newly-synthesised Ii polypeptides also oligomerise, forming homotrimeric complexes *via* interchain disulphide bridges in their cytoplasmic tails between residues, 163–183 (Figure 1.6; Biljmakers *et al.*, 1994a; Bertolino *et al.*, 1995). Each of these Ii trimers represents a scaffold onto which three αβ class II MHC dimers may assemble (Roche *et al.*, 1991; Marks *et al.*, 1995a) in a process facilitated by the association of the ER-resident protein, calnexin (Anderson & Cresswell, 1994; Schreiber *et al.*, 1994; Arunachalam & Cresswell, 1995; Romagnoli & Germain, 1995; Williams & Watts, 1995). Each individual $(\alpha\beta Ii)_3$ nonamer may contain non-identical class II MHC heterodimers as a result of the multiple isotypes and allotypes expressed within class II MHC-positive cells. Once assembled, these multimeric, high molecular weight complexes are transported into the Golgi complex at which location they undergo glycosylation and terminal sialylation.

The association of Ii with the class II MHC molecules in the ER has a number of important functions. In support of this, Ii-deficient (Ii^0) mice exhibit significantly reduced presentation of antigens by class II MHC molecules, defective B cell development and impaired positive selection of $CD4^+$ T cells (*e.g.* Bikoff *et al.*, 1993; Viville *et al.*, 1993; Naujokas *et al.*, 1995; Shachar & Flavell, 1996; Wong & Rudensky, 1996). Firstly, the initial assembly of the αβ heterodimers onto the Ii trimer scaffold is critical for stabilising the class II MHC molecules in a correctly-folded state for their egress from the ER (Roche *et al.*, 1991). In the absence of Ii, αβ heterodimers

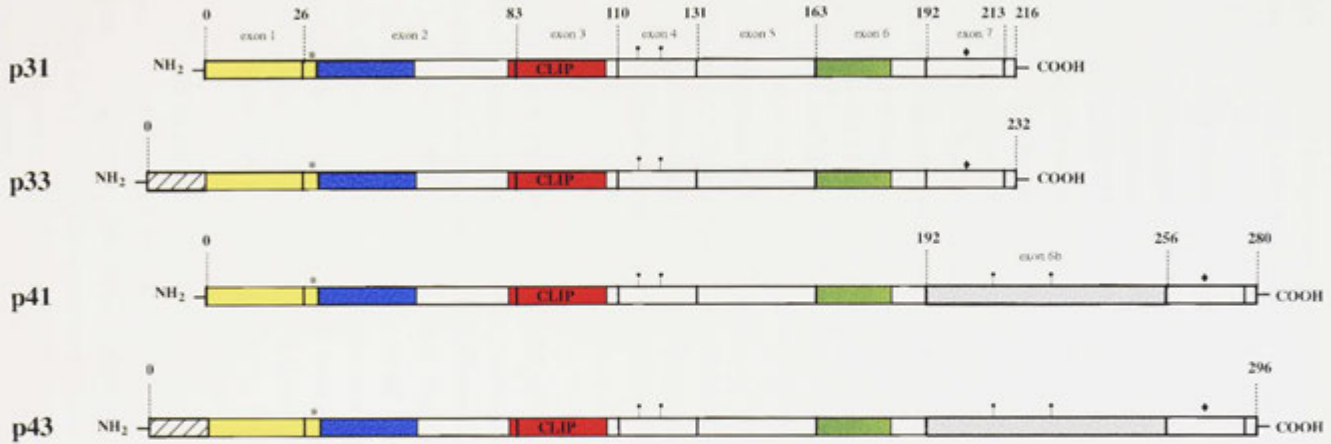


Figure 1.6 *The structural organisation of the invariant chain protein.*

Human invariant chain exists in four isoforms resulting from an alternative translation initiation codon and the alternate splicing of the 6b exon. The 16-residue N-terminal extension is indicated by cross-hatching. The alternatively-spliced 64-amino acid element encoded within exon 6b is shaded. Numbering on the p31 Ii marks the exon boundaries. Also indicated are the cytoplasmic domain (yellow), transmembrane domain (blue), CLIP region (red) and trimerisation signal (green). The site of palmitic acid addition (Cys 28) is marked with an asterisk. The chondroitin sulphate addition at Ser 202 (p31 numbering) is marked with a diamond. Putative glycosylation sites are indicated by lollipops. See text for references.

accumulate intracellularly and are poorly terminally-glycosylated (Schaiff *et al.*, 1991, 1992; Anderson & Miller, 1992; Elliott *et al.*, 1994a). This observation also illustrates the second role for Ii, that of assisting the class II MHC molecules to leave the ER and travel through the Golgi complex to the *trans*-Golgi network (TGN). Here, the αβIi complexes are sorted and diverted from the default secretory pathway into the endocytic route by virtue of two leucine-based localisation signals mapped to the N-terminal cytoplasmic tail of Ii (Bakke & Dobberstein, 1990; Lotteau *et al.*, 1990). One of these motifs has been identified to involve the residues, Leu 7 and Ile 8 (Pieters *et al.*, 1993; Figure 1.5). The other is located between Pro 15–Leu 17 (Bremnes *et al.*, 1994; Odorizzi *et al.*, 1994). Each is reported to require an acidic amino acid 4–5 residues N-terminal to the leucine motif (Pond *et al.*, 1995). These or other signals may also act to retain the class II MHC molecules within this location until their Ii chaperone is degraded with concomitant loading of antigen-derived peptide (Loss & Sant, 1993; Gorvel *et al.*, 1995). The extent to which αβIi complexes reach the endosomes *via* internalisation from the cell surface *versus* direct trafficking from the TGN is still unresolved but may depend upon the cell type (*e.g.* Roche *et al.*, 1993; Simonsen *et al.*, 1993; Benaroch *et al.*, 1995; Swier & Miller, 1995a; Salamero *et al.*, 1996; Wang *et al.*, 1997; Kang *et al.*, 1998; Liu *et al.*, 1998; Saudrais *et al.*, 1998; Ong *et al.*, 1999). A similar course may also be taken by some class II MHC molecules in Ii° cells, possibly by way of a signal within a highly-conserved region of the class II MHC β-chain, residues 80–83 (Chervonsky *et al.*, 1994; Tan *et al.*, 1997a).

The third function of Ii is to prevent class II MHC αβ dimers from forming premature associations with antigen-derived peptides both within the ER and while in transit to the endosomes (Roche & Cresswell, 1990b, 1991; Teyton *et al.*, 1990; Demotz, 1993; Newcomb & Cresswell, 1993; Long *et al.*, 1994). Similarly, this interaction thwarts the binding of intact, endogenous polypeptides in the ER (Busch *et al.*, 1995, 1996). This feature of the Ii protein involves the exon 3-encoded region, residues 82–107 (Figure 1.6; Romagnoli & Germain, 1994). The deletion of these residues abrogates the binding of Ii to class II MHC molecules, confirming this as the principle site of contact between these proteins (Freisewinkel *et al.*, 1993; Biljmakers *et al.*, 1994a).

In the absence of αβ heterodimers in human cells, Ii trimers are retained in the ER by a double arginine retention signal in the N-terminal extension shared by the p33 and p43 forms (Figure 1.6; Marks *et al.*, 1990; Arunachalam *et al.*, 1994; Schutze *et al.*, 1994). The p31 and p41 forms of Ii lack this signal but may also be retained through the formation of mixed trimers, containing at least one Ii subunit with the ER retention signal (Lamb & Cresswell, 1992).

1.5. Formation of antigenic peptide–class II MHC complexes

Unlike class I MHC molecules, which reach the cell surface within minutes after their arrival in the TGN, there is a 1–3 hour delay between the appearance of class II MHC molecules in the TGN and their deposition at the cell surface (Neefjes *et al.*, 1990). During this time, the αβIi complexes are targeted to compartments in the endocytic pathway where Ii is degraded and antigen-derived ligand is bound.

1.5.1 The proteolytic degradation of Ii

Before class II MHC molecules may bind antigen-derived peptides for presentation to $CD4^+$ T cells at the cell surface, the αβ heterodimers must first be released from their interaction with Ii (Roche *et al.*, 1992). This is achieved through the step-wise removal of fragments of the Ii protein generated by proteolytic cleavage (Maric *et al.*, 1994).

The proteolysis of Ii occurs *via* three major degradation intermediates, all of which remain complexed with the class II MHC αβ dimers: the 21–22 kDa leupeptin-induced polypeptides ('LIP'; Blum & Cresswell, 1988), the small leupeptin-induced peptides ('SLIP') of 10–14 kDa (Nguyen *et al.*, 1989; Maric *et al.*, 1994) and, lastly, the nested set of class II MHC-associated invariant chain peptides or 'CLIP' (Rudensky *et al.*, 1991; Chicz *et al.*, 1992, 1993; Hunt *et al.*, 1992; Riberdy *et al.*, 1992; Sette *et al.*, 1992a). The LIP fragments correspond to the N-terminal two-thirds of the Ii protein including the cytoplasmic and transmembrane regions, the signals responsible for sorting and retention and the exon 3-derived region necessary for interaction with the αβ dimer (Figure 1.6). Cleavage of the intact Ii to produce LIP is proposed to occur between residues Leu 174–Phe 175, a bond conserved in human, mouse, rat and cow Ii (Figure 1.5; Kageyama *et al.*, 1996). The SLIP fragments are produced when LIP is cleaved approximately between Ii residues 105 and 106 (Chapman, 1998). This releases the individual αβIi trimers from their nonameric complex. The cleavage of SLIP then yields αβ class II MHC dimers complexed with CLIP (Xu *et al.*, 1994; Amigorena *et al.*, 1995; Morkowski *et al.*, 1995; Kageyama *et al.*, 1996). These last Ii fragments are derived almost exclusively from the exon 3-encoded Ii moiety (residues 82–107) shown to be critical for the Ii-class II interaction and which blocks the premature binding of antigen-derived peptides (Freisewinkel *et al.*, 1993; Romagnoli & Germain, 1994).

The bulk of Ii proteolysis occurs within the later parts of the endocytic pathway, involving acidic proteases (Blum & Cresswell, 1988; Roche & Cresswell, 1991). The identification of the key proteases involved in the production of LIP, SLIP and CLIP has been complicated by the high degree of functional redundancy that exits amongst such enzymes within these cellular compartments. Studies in B-lymphoblastoid cell lines (B-LCL) using protease inhibitors have demonstrated that Ii proteolysis in these APC may be initiated by an aspartyl proteinase (Maric *et al.*, 1994). Enzymes of this nature, such as the cathepsins D and E, are found in high concentration in lysosomal compartments and are optimally active at acidic pH. The cysteine proteinase, cathepsin B, has also been implicated in the generation of the LIP and SLIP fragments (Reyes *et al.*, 1991; Roche & Cresswell, 1991; Mizuochi *et al.*, 1994; Xu *et al.*, 1994). This enzyme exhibits both endo- and dipeptidyl-peptidase activity and is active at both acidic and neutral pH. However, APC from knockout mice that are genetically-deficient in either cathepsin B or D expression show no obvious defects in class II MHC antigen presentation (Villadangos *et al.*, 1997; Deussing *et al.*, 1998).

By contrast, an essential role in the degradation of Ii has been shown for the cysteine protease, cathepsin S. This enzyme is believed to catalyse the final cleavage of the SLIP fragment to produce the CLIP–class II MHC complexes (Riese *et al.*, 1996). Two potential cleavage sites are the bonds, Arg 78–Met 79, or Lys 80–Leu 81, both conserved in mouse, rat, human and cow Ii and consistent with the known substrate preferences of cathepsin S (Figure 1.5; Riese *et al.*, 1996). The disruption of cathepsin S activity in APC prevents the complete proteolysis of both human and mouse Ii, resulting in the accumulation of $\alpha\beta$ dimers complexed with 10–13 kDa SLIP-like fragments and the inhibition of class II MHC-mediated antigen presentation (Riese *et al.*, 1996, 1998; Villadangos *et al.*, 1997; Nakagawa *et al.*, 1999). Mice lacking expression of this enzyme exhibit defective humoral immune responses (Shi *et al.*, 1999). Cathepsin S is expressed predominantly in the major APC types and is a potent endopeptidase active over a broad pH range (Shi *et al.*, 1992, 1994; Bromme *et al.*, 1993). The inhibition of this enzyme by the endogenous cysteine protease inhibitor, cystatin C, has been suggested to be a means by which antigen presentation by class II MHC molecules is controlled during the maturation of dendritic cells (Pierre & Mellman, 1998; Driessen *et al.*, 1999).

Sequence features of the Ii protein may influence its own proteolysis and that of other proteins. The amino-terminal region of Ii shares 50% sequence homology with members of the cystatin superfamily and may inhibit the activity of cathepsins L and H (Katunuma *et al.*, 1994). In addition, the alternatively-spliced segment of the p41 isoform of Ii is a

member of the thyroglobulin type-1 domain class of cysteine protease inhibitors (Lenarcic & Bevec, 1998). This Ii fragment shows potent inhibition of cathepsin L (Ogrinc *et al.*, 1993; Bevec *et al.*, 1996; Fineschi *et al.*, 1996) by binding within the active site cleft in a novel wedge-shaped fold that is stabilised by three internal disulphide bonds between cysteine residues, 197–216, 227–234 and 236–255 (Figure 1.5; Guncar *et al.*, 1999). This may account for the ability of the p41 Ii isoform to enhance the presentation of a particular subset of antigens (Peterson & Miller, 1992), for example, by preventing the proteolytic destruction of some epitopes. Similarly, p41 itself has a longer half-life in the endosomal compartments than p31 (Arunachalam *et al.*, 1994) and a 12 kDa SLIP-like fragment exhibits a prolonged association with the class II MHC molecules (Fineschi *et al.*, 1995, 1996). However, these two Ii isoforms display largely equivalent effects upon other aspects of class II MHC-mediated presentation of antigens, including subunit assembly, endosomal localisation, peptide acquisition, cell-surface expression and $CD4^+$ T cell development (Shachar *et al.*, 1995; Takaesu *et al.*, 1995, 1997; Serwe *et al.*, 1997; Wright *et al.*, 1998). Inhibition of cathepsin S by the p41 fragment is unlikely as a result of a number of possible unfavourable electrostatic interactions (Guncar *et al.*, 1999).

1.5.2 The mediation of peptide loading by HLA-DM/H-2M

A key step in the presentation of antigen by class II MHC molecules is the release of the $\alpha\beta$ dimers from their association with CLIP. Only once these final proteolytic fragments of Ii have been displaced may the individual molecules bind antigen-derived peptides (Freisewinkel *et al.*, 1993; Romagnoli & Germain, 1994). This process is facilitated by another MHC-encoded heterodimer, HLA-DM (H-2M in the mouse).

HLA-DM is a non-classical MHC molecule encoded by the genes *DMA* and *DMB* which display only limited allelic polymorphism (Figure 1.1a; Carrington *et al.*, 1993; Sanderson *et al.*, 1994b). The homologous genes in the mouse are designated *Ma* and *Mb* and are similarly non-polymorphic (Figure 1.1b; Hermel *et al.*, 1995). The α- and β-chains exhibit 25–30% sequence identity to those of the classical class II MHC heterodimers and the molecules share similar domain arrangements, including the formation of a membrane-distal peptide-binding groove (Figure 1.3; Cho *et al.*, 1991; Kelly *et al.*, 1991). However, in HLA-DM and H-2M this site is not capable of accommodating a peptide ligand. The α-helices of the α_1 and β_1 domains are packed closely together so as to occlude all but a single pocket (Fremont *et al.*, 1998a; Mosyak *et al.*, 1998). The molecules are also thought to be more rigid due to the formation of two

disulphide bonds additional to those found in the classical peptide-receptive class II MHC molecules (Figure 1.3).

A role for HLA-DM in the class II MHC-mediated pathway of antigen presentation was first proposed from the finding that a deficiency in the expression of this molecule was responsible for the aberrant functioning of a set of mutant APC, generated by ethyl methane sulphonate (EMS) treatment of an EBV-transformed human B lymphoblastoid cell line, 8.1.6 (Kelly *et al.*, 1991; Mellins *et al.*, 1991; Denzin *et al.*, 1994; Fling *et al.*, 1994; Morris *et al.*, 1994). These mutants had been characterised by their loss of the conformation-sensitive epitope recognised by the anti-HLA-DR3 monoclonal antibody, 16.23 (Pious *et al.*, 1985) and were unable to present determinants derived from native protein antigen, although capable of presenting exogenously-added peptide (Mellins *et al.*, 1990). The cells had intact structural genes of both Ii and class II MHC molecules and expressed normal levels of each. However, the HLA-DR molecules were unstable in SDS detergent and cell-surface αβ dimers were loaded predominantly with the Ii peptides, CLIP (Riberdy *et al.*, 1992; Sette *et al.*, 1992a; Monji *et al.*, 1994). A similar phenotype has been observed subsequently for APC from H-2M knockout mice (Fung-Leung *et al.*, 1996; Martin *et al.*, 1996; Miyazaki *et al.*, 1996).

HLA-DM is now recognised to be able to facilitate the binding of antigen-derived peptides to class II MHC αβ dimers by inducing the dissociation of pre-existing ligands from these molecules (The following details are also applicable to mouse H-2M). Susceptible peptides include but are not limited to CLIP (Denzin & Cresswell, 1995; Sherman *et al.*, 1995; Sloan *et al.*, 1995; van Ham *et al.*, 1996) and possibly also the larger Ii fragments, LIP and SLIP (Denzin & Cresswell, 1995; Denzin *et al.*, 1996; Schafer *et al.*, 1996). The sensitivity of a given peptide–class II MHC complex to the actions of HLA-DM is determined by its overall kinetic stability, such that this molecule enhances the dissociation rate of all peptides but to an extent directly proportional to their intrinsic rate of dissociation (Kropshofer *et al.*, 1996; Weber *et al.*, 1996). HLA-DM also stabilises the empty class II MHC heterodimer so generated against functional inactivation and aggregation until a more stable interaction has formed with another peptide (Denzin *et al.*, 1996; Kropshofer *et al.*, 1997; Kovats *et al.*, 1998). In this way, HLA-DM is able to influence the array of peptides presented at the cell surface to T lymphocytes by skewing the population towards those that form long-lived complexes with class II MHC molecules (Sloan *et al.*, 1995; Katz *et al.*, 1996; Lightstone *et al.*, 1997).

The expression of HLA-DM closely parallels that of conventional class II MHC molecules. *HLA-DM* gene products are expressed constitutively in APC under the

influence of the class II MHC transactivator (CIITA; Chang & Flavell, 1995) and may be induced in other tissues by IFN-γ (Kelly *et al.*, 1991). However, the molar ratio of HLA-DM to class II MHC molecules *in vivo* is less than 1:5 (Schafer *et al.*, 1996). Consistent with this, HLA-DM is active in substoichiometric quantities *in vitro* and a single molecule is able to convert 3–12 CLIP–HLA-DR complexes *per* minute (Vogt *et al.*, 1996).

The mediation of peptide loading of class II MHC molecules by HLA-DM takes place optimally between pH 4.5–5.5 (Denzin & Cresswell, 1995; Sherman *et al.*, 1995; Sloan *et al.*, 1995). This is compatible with the intracellular accumulation of these molecules within the mildly acidic, class II MHC-enriched vesicles of the endocytic route (Denzin *et al.*, 1994; Karlsson *et al.*, 1994; Sanderson *et al.*, 1994a; Green *et al.*, 1995; Robbins *et al.*, 1996). Both HLA-DM and H-2M are targeted to these compartments by virtue of a tyrosine-based motif in the cytoplasmic tail of β-chain (Lindstedt *et al.*, 1995; Marks *et al.*, 1995b), similar to that of the lysosomal-associated membrane protein, LAMP-1, which co-resides intracellularly with these molecules (Williams & Fukuda, 1990; Copier *et al.*, 1996). HLA-DM is found infrequently at the cell surface, consistent with this consensus sequence signalling rapid internalisation from the plasma membrane in clathrin-coated pits (Ktistakis *et al.*, 1990). In this absence of this motif, Ii may direct HLA-DM/H-2M into the appropriate endocytic compartments (Lindstedt *et al.*, 1995; Copier *et al.*, 1998).

The molecular mechanism by which HLA-DM is believed to facilitate peptide exchange from class II MHC αβ dimers involves the transient association of these molecules. Such an interaction has been observed by co-immunoprecipitation (Denzin *et al.*, 1996; Sanderson *et al.*, 1996). The inhibition of HLA-DM activity by either the addition of an N-linked glycan to position 94 on the α_2 domain of the HLA-DR3 heterodimer (Sanderson *et al.*, 1996; Guerra *et al.*, 1998) or the binding of the CerCLIP.1 antibody to the N-terminus of CLIP complexed with HLA-DR3 (Denzin & Cresswell, 1995) implicates a region on a lateral face of the class II MHC molecule as a potential site of contact. The interaction between HLA-DM and HLA-DR may take place through exposed hydrophobic domains, each shielding the other from the aqueous environment. Under acidic conditions approximating those of the endocytic compartments, both HLA-DM and HLA-DR molecules undergo conformational changes that lead to the exposure of such non-polar regions (Runnels *et al.*, 1996; Ullrich *et al.*, 1997; Busch *et al.*, 1998). In this environment, the HLA-DM molecule may stabilise a more open conformation of the class II MHC peptide-binding groove that favours the rapid dissociation and association of peptides. HLA-DM is proposed to dissociate when the available interactive surface is

reduced as a result of a conformational change in the class II MHC αβ dimer upon its forming a stable association with a peptide (Ullrich *et al.*, 1997).

1.5.3 HLA-DO, a regulator of HLA-DM activity

Another molecular component of the class II MHC-mediated antigen presentation pathway is HLA-DO (H-2O in the mouse), the product of the genes, *DNA* and *DOB* (*Oa* and *Ob* (formerly *Aβ2*) in the mouse; Figure 1.1; Larhammar *et al.*, 1985; Tonnelle *et al.*, 1985; Trowsdale & Kelly, 1985; Servenius *et al.*, 1987; Karlsson & Peterson, 1992). Like HLA-DM, this molecule is a non-classical class II MHC heterodimer that displays minimal allelic polymorphism. HLA-DO resides intracellularly in stable complexes with HLA-DM within the endocytic compartments (Liljedahl *et al.*, 1996; Denzin *et al.*, 1997). This association is required for the efficient exit of HLA-DO from the ER, despite the presence of two endosomal localisation signals within its β-chain (Samaan *et al.*, 1999). A population of H-2O has also been described complexed with Ii (Karlsson *et al.*, 1991; Douek & Altmann, 1997).

Although much still remains to be learned of HLA-DO, this molecule would appear to regulate the stringency with which HLA-DM facilitates peptide exchange (Denzin *et al.*, 1997; van Ham *et al.*, 1997; Kropshofer *et al.*, 1998; Liljedahl *et al.*, 1998). Complexes of HLA-DO and HLA-DM have been isolated in association with HLA-DR molecules (Kropshofer *et al.*, 1998). HLA-DO is able to enhance the ability of HLA-DM to stabilise empty class II MHC αβ dimers (Kropshofer *et al.*, 1998) and may influence the pH range over which HLA-DM is active (Liljedahl *et al.*, 1998). Studies of H-2O-deficient mice suggest that this molecule may also serve to promote the presentation of particular antigens, for example, those internalised into B cells by their membrane immunoglobulin receptors *versus* those captured by fluid-phase endocytosis (see below, §1.5.4; Liljedahl *et al.*, 1998).

The possibility that HLA-DO may regulate the activities of HLA-DM is particularly interesting in light of its unusual tissue distribution. Mouse H-2O is expressed in B cells and in a subset of thymic medullary epithelial cells, independently of the presence of classical class II MHC molecules. This molecule has not been detected in dendritic cells and macrophages (Wake & Flavell, 1985; Karlsson *et al.*, 1991). Human HLA-DO has a similar pattern of expression but is also present in some dendritic cell populations (Douek & Altmann, 1997). The expression of HLA-DO is not induced by the CIITA like many other MHC gene products (Tonnelle *et al.*, 1985; Denzin *et al.*, 1997) and the *Ob* gene is

not sensitive to upregulation by IFN-γ (Wake & Flavell, 1985). *HLA-DOB* is inducible by IFN-γ but the mRNA is unstable (Ponzoni *et al.*, 1993). Together, these findings suggest that HLA-DO/H-2O may have a critical role in regulating the specific peptide repertoire presented by these particular APC.

1.5.4 The processing of protein antigens for presentation by class II MHC molecules

Prior to presentation by class II MHC molecules at the cell surface, protein antigens must enter the endocytic route and undergo processing. The internalisation of proteins may occur by a number of different means (reviewed by Lanzavecchia, 1990 and Watts, 1997). Random samples of extracellular proteins may be engulfed by fluid-phase pinocytosis. Macrophages and dendritic cells capture particulate antigen contained within immune complexes by phagocytosis. B cells use the membrane immunoglobulins (mIg) of the multimeric B cell receptor (BCR) to mediate the highly-efficient uptake of specific antigens.

Upon internalisation, the antigen typically travels down a progressively decreasing pH gradient through the early and late endosomes before reaching the lysosomes (pH 4.5–5.0), the major site of intracellular proteolysis (Harding & Geuze, 1993). Antigens bound to the BCR may exhibit accelerated targeting into class II MHC-enriched compartments (Mitchell *et al.*, 1995). The extent of processing required for a given antigen to be presented by a class II MHC molecule will depend upon the spatial environment of T cell determinants within the molecule. For example, an epitope within a tight globular protein may require proteolytic cleavage to be released from its steric constraints, whereas others may require simply the unfolding of the polypeptide. Antigen processing has been defined as any change in the protein which affords it the conformational flexibility to be able to interact with both the MHC peptide-binding site and, subsequently, the TCR (Allen, 1987). The size of a protein fragment does not dictate the need for processing and intact proteins may bind to class II MHC molecules *via* exposed determinants (Streicher *et al.*, 1984; Lindner & Unanue, 1996; Arunachalam *et al.*, 1998).

The presence of an MHC-binding determinant within a protein is not sufficient to guarantee its presentation at the cell surface. In addition to ease of access, the specificity of the proteolytic enzymes encountered will have a major influence upon the immunodominance of different epitopes. Both exo- and endo-proteolytic activities

present within the acidic compartments of the endocytic route, including the aspartyl proteases (*e.g.* cathepsins D and E) and the cysteine proteases (*e.g.* cathepsins B, L and S; reviewed by Watts, 1997; Chapman, 1998 and McGrath, 1999). Additional enzymes within this system are still being discovered (Manoury *et al.*, 1998).

1.5.5 The effect of pH on the interaction of peptides with class II MHC molecules

The rate of association of a peptide with a class II MHC molecule is unusually slow for a ligand–receptor interaction (Buus *et al.*, 1986). Binding is proposed to proceed *via* a short-lived kinetic intermediate before undergoing a slow structural transition (Sadegh-Nasseri & McConnell, 1989). Once formed, the terminal peptide–αβ complexes are extremely stable with half-lives ranging from several hours to days (Buus *et al.*, 1986; Roche & Cresswell, 1990a). This is of considerable functional significance in allowing each peptide-bound class II MHC molecule to be surveyed adequately at the cell surface by the T cell population.

In addition to the actions of HLA-DM (§1.5.2), the formation of stable peptide–MHC complexes *in vivo* is facilitated by high proton concentration. The effect of pH on this reaction was first proposed to explain discrepancies observed between the efficient presentation of antigen *in vivo* (Harding *et al.*, 1989; Davidson *et al.*, 1991) and the slow rate of complex formation at neutral pH *in vitro* (Buus *et al.*, 1986; Jensen, 1990). Agents that raised the pH in the endocytic compartments, such as ammonium chloride and chloroquine, inhibited efficient antigen processing (Ziegler & Unanue, 1982) and the formation of functional antigen–class II MHC complexes was accelerated markedly *in vitro* at pH values approximating those of the intracellular endosomal vesicles (Jensen, 1990, 1992; Sadegh-Nasseri & Germain, 1991; Tampé & McConnell, 1991; Wettstein *et al.*, 1991).

An optimal rate of complex formation is achieved typically between pH 4.5–6.0 (Jensen, 1990; Harding *et al.*, 1991; Mouritsen *et al.*, 1992; Sette *et al.*, 1992b). The optimum pH depends on both the particular peptide and class II MHC molecule under consideration (Wettstein *et al.*, 1991; Sette *et al.*, 1992b). As a result, individual class II MHC αβ dimers have been proposed to load their peptide cargo in different locations within the endocytic pathway, as best suits their pH binding optima (Jensen, 1991; Sette *et al.*, 1992b). The exposure of different allotypes to distinct pools of peptides may be a means by which to broaden the peptide repertoire presented at the cell surface.

High proton concentration appears to accelerate not only the association of peptides with class II MHC molecules, but also the rate at which complexes dissociate (Sadegh-Nasseri & Germain, 1991; Tampé & McConnell, 1991; Reay *et al.*, 1992; Sette *et al.*, 1992b). This may still have the net result of enhancing the peptide-binding capacity by increasing the pool of active, unoccupied receptor sites available (Jensen, 1991; Sette *et al.*, 1992b). The low-pH enhancement of both the association and dissociation rates of a peptide–αβ complex may arise by such conditions increasing the conformational flexibility of the molecule to render the peptide-binding groove more accessible. Conformational changes have been detected in class II MHC proteins at acidic pH (Lee *et al.*, 1992; Boniface *et al.*, 1996; Runnels *et al.*, 1996; Reich *et al.*, 1997; Ullrich *et al.*, 1997).

1.5.6 The intracellular site of peptide–class II MHC complex formation, the MIIC

Numerous studies have implicated the acidic compartments of the endocytic pathway as the site at which class II MHC molecules and peptides interact (Ziegler & Unanue, 1982; Harding *et al.*, 1990; Jensen, 1990, 1991; Neefjes *et al.*, 1990; Wettstein *et al.*, 1991; Sette *et al.*, 1992b). However, the exact location within this intracellular network at which peptide–αβ complexes form has been difficult to pinpoint. Using the techniques of density-gradient electrophoresis (Tulp *et al.*, 1994), immunoelectron microscopy (Harding & Geuze, 1993) and percoll-density gradient centrifugation (Qiu *et al.*, 1994; West *et al.*, 1994), the majority of class II MHC molecules have been identified to accumulate in a group of intracellular compartments, designated collectively the MIIC for 'MHC class II compartments'. The conflicting morphology and marker profiles of the MIIC reported by different studies supports the notion that these are not specialised endocytic structures but, rather, a heterogeneous set of acidic vesicles exhibiting features of both late endosomes and early lysosomes (reviewed by Geuze, 1998 and Neefjes, 1999). MIIC have been identified in all types of professional APC (Harding & Geuze, 1993; Kleijmeer *et al.*, 1995; Nijman *et al.*, 1995; Peters *et al.*, 1995).

Newly-synthesised αβIi nonamers may enter the endocytic route from the TGN at the level of the transferrin receptor-positive (TfR^+) early endosomes (Romagnoli *et al.*, 1993; Castellino & Germain, 1995; Pond & Watts, 1999). However, the early endosomes are unlikely to be the sites of class II MHC peptide loading due to both their minimal proteolytic activity and lack of HLA-DM (Peters *et al.*, 1995; Fernandez-Borja *et al.*, 1996). Instead, αβIi complexes progress to later endocytic vesicles in a process facilitated by the actin cytoskeleton (Barois *et al.*, 1998). The first significant proteolytic

sites to be accessed by these complexes are the early MIIC. Some αβIi may also enter these compartments directly from the TGN (Lotteau *et al.*, 1990; Neefjes *et al.*, 1990; Peters *et al.*, 1991, 1995). The early MIIC are TfR^+ like the early endosomes but also exhibit evidence of Ii degradation through the progressive disappearance of both lumenal and cytoplasmic epitopes of anti-Ii antibodies (Tulp *et al.*, 1994; Kleijmeer *et al.*, 1997). These compartments fit the description of the 'class II MHC-containing vesicles' (CIIV) identified by free-flow electrophoresis in the mouse B cell line, A20 (Amigorena *et al.*, 1994). Further Ii degradation takes place within the intermediate MIIC which are a collection of TfR^- compartments characterised by the presence of abundant internal vesicles and the late endosomal marker, the mannose-6-phosphate receptor (MPR; Kleijmeer *et al.*, 1994, 1996; Peters *et al.*, 1995; Xu *et al.*, 1995; Morkowski *et al.*, 1997; Stang *et al.*, 1998). Finally, the intermediate MIIC mature into the late MIIC. These are dense, multilaminar vesicles, positive for the lysosomal markers, cathepsin D, CD63, LAMP-1 and β-hexosaminidase, but negative for MPR, TfR and invariant chain (Kleijmeer *et al.*, 1994, 1996; Peters *et al.*, 1995). All subclasses of the MIIC are enriched for class II MHC and HLA-DM (Sanderson *et al.*, 1994a; Fernandez-Borja *et al.*, 1996; Kleijmeer *et al.*, 1997; Morkowski *et al.*, 1997; Tan *et al.*, 1997b) and may be accessed by antigen internalised through membrane Ig, consistent with these being the major intracellular sites of class II MHC maturation (West *et al.*, 1994). Peptide-loaded class II MHC molecules are detected in all MIIC but to the greatest extent in the late MIIC (Castellino & Germain, 1995; Kleijmeer *et al.*, 1997; Morkowski *et al.*, 1997; Stang *et al.*, 1998).

1.5.7 Deposition of peptide–αβ complexes on the cell surface

Once antigen-derived peptides have been loaded into the groove of class II MHC molecules in the MIIC, the complexes must migrate to the cell surface to present their cargo to the T cell repertoire. The TfR^+ early MIIC and CIIV may recycle their contents back to the plasma membrane directly by means of the conventional receptor-recycling pathway. However, for the TfR^- intermediate and late MIIC, alternative mechanisms must exist.

One possibility is that assembled peptide–class II MHC complexes from the later MIIC might exhibit retrograde transport through the endocytic route and exploit the recycling machinery of the early endosomes. However, peptide-loaded class II MHC molecules are still able to reach the surface of B cells after these compartments have been ablated, suggesting that this is not the predominant mechanism (Pond & Watts, 1997).

Alternatively, the MIIC may fuse directly with the plasma membrane. Protein markers of the MIIC, such as LAMP-1 and CD63, have been found in indentations on the surface of B cells, co-localised with class II MHC molecules (Raposo *et al.*, 1996). These molecules would be expected to be internalised soon after reaching this site by virtue of the targeting signals in their cytoplasmic tails (Williams & Fukuda, 1990; Metzelaar *et al.*, 1991). The limiting membranes of class II MHC-positive vesicles have also been visualised fusing with the plasma membrane (Wubbolts *et al.*, 1996). However, a kinetic analysis of this process suggests that it too is unlikely to be the major mechanism by which mature peptide–αβ complexes are delivered to the cell surface (Raposo *et al.*, 1996). Another possibility is the existence of specialised transport vesicles. These would require some form of yet-uncharacterised sorting event in the MIIC. Candidate class II MHC-enriched vesicles derived from phagolysosomes have been identified in macrophages (Harding & Geuze, 1992).

1.6. Deviations from the classical pathway of class II MHC antigen presentation

1.6.1 Class II MHC-mediated presentation of endogenous peptides

Although the peptide repertoire sampled by class II MHC αβ dimers in the MIIC is derived predominantly from internalised extracellular proteins (§1.5.4), an examination of the pool of naturally-processed peptides presented by these molecules reveals that some ligands originate from proteins that reside within the cell (Nelson *et al.*, 1992; Chicz *et al.*, 1993, 1994; Newcomb & Cresswell, 1993; Rötzschke & Falk, 1994; Harris *et al.*, 1996; Godkin *et al.*, 1997). These may include viral antigens which have been synthesised during an infection (Sekaly *et al.*, 1988; Jacobson *et al.*, 1989; Jaraquemada *et al.*, 1990; Nuchtern *et al.*, 1990; Malnati *et al.*, 1992, 1993; Khanna *et al.*, 1997; Oxenius *et al.*, 1997). In many cases, intracellular proteins are available to bind to class II MHC molecules simply by being natural inhabitants of the endocytic pathway. However, others are localised elsewhere within the cell with no obvious means of accessing the MIIC (*e.g.* Brooks *et al.*, 1991; Brooks & McCluskey, 1993; Loss *et al.*, 1993). This has been termed the endogenous pathway of class II MHC antigen presentation (reviewed by Lechler *et al.*, 1996).

It is possible that determinants within some cytosolic proteins might gain access to class II MHC molecules in the endocytic route when the proteins are degraded in the lysosomes at the end of their useful lifespan, for example, transported *via* autophagic vacuoles (Dunn,

1990; Seglen & Bohley, 1992; Liou *et al.*, 1997) or by binding to specific receptors (Chiang *et al.*, 1989; Cuervo & Dice, 1996). Others may enter these sites when entire dead cells are engulfed by phagocytosis.

An alternative mechanism is that class II MHC αβ dimers may bind antigen in the ER prior to the interaction of Ii. This may involve either small protein fragments or partially-folded proteins which are subsequently trimmed (Weiss & Bogen, 1991; Biljmakers *et al.*, 1994b; Busch *et al.*, 1995, 1996; Lechler *et al.*, 1996). Under normal conditions, this process is likely to involve only a minority of class II MHC molecules due to the molar excess of Ii present in the ER, its faster on-rate than that of antigen-derived peptides and the neutral pH within this site (Biljmakers *et al.*, 1994b; Bodmer *et al.*, 1994; Busch *et al.*, 1995). However, this phenomenon may be of some significance if the ratio of Ii to class II MHC molecules is altered, for example, during an inflammatory response or in the context of high antigen load during a viral infection. A population of class II MHC molecules form complexes with peptides and polypeptides in the ER of Ii° cells (Busch *et al.*, 1995, 1996; Lechler *et al.*, 1996).

Class I MHC molecules may also deviate from their classical pathway of antigen presentation and display peptides of extracellular origins (*e.g.* Carbone & Bevan, 1990; Rock *et al.*, 1990; Kovacsovics-Bankowski *et al.*, 1993; Pfeifer *et al.*, 1993). In this respect, it is interesting to note that some empty class I MHC heterodimers are able to associate with Ii (Cerundolo *et al.*, 1992; Sugita & Brenner, 1995; Vigna *et al.*, 1996) and this interaction has been found to direct these molecules into the endocytic route (Sugita & Brenner, 1995).

1.6.2 Role of class II MHC receptor recycling in antigen presentation

Similar to other cell-surface receptors, peptide-bound class II MHC molecules on the plasma membrane may be internalised periodically into the early endosomes (Pernis, 1985; Schmid *et al.*, 1988; Machy *et al.*, 1990; Reid & Watts, 1990; Salamero *et al.*, 1990; Swier & Miller, 1995b). This process appears to be controlled by signal motifs located in each cytoplasmic tail of the αβ dimer (Pinet *et al.*, 1995; Zhong *et al.*, 1997; Forquet *et al.*, 1999). Within the early endosomes, the recycling class II MHC molecules encounter new antigens and may undergo peptide exchange (Adorini *et al.*, 1989; Harding *et al.*, 1990; Reid & Watts, 1990; Pedrazzini *et al.*, 1991; Griffin *et al.*, 1997; Pinet & Long, 1998). This represents a mechanism of ligand acquisition distinct from that involving the newly-synthesised αβ dimers in the MIIC (§1.5.6; Davidson *et al.*, 1991).

Only a minority of recycling class II MHC molecules are candidates for reuse. Most complexes formed between antigen-derived peptides and class II MHC molecules are very stable with half-lives approximating those of the αβ dimers themselves (*e.g.* Lanzavecchia, 1987; Jensen, 1989, 1992; Lee & Watts, 1990; Lanzavecchia *et al.*, 1992; Bot *et al.*, 1996; Monji & Pious, 1997). Such molecules are unlikely to present more than one peptide antigen during their life-time. However, other peptide–class II MHC combinations have a higher rate of turnover (*e.g.* Ceppellini *et al.*, 1989; Harding *et al.*, 1989; Adorini & Nagy, 1990a; Tampé & McConnell, 1991; Marsh *et al.*, 1992; Muller *et al.*, 1993; Poirier & Chain, 1993; Nelson *et al.*, 1994). A population of class II MHC molecules associated with low affinity peptides, or even empty, has been detected on the surface of B cells *in vivo* (Germain & Hendrix, 1991). Peptide exchange by these molecules during recycling may enable more stable determinants to be presented to T cells. The displacement of one ligand in favour of a strong T cell responder has been observed *in vitro* (Reay *et al.*, 1992).

A number of class II MHC-restricted antigenic determinants have now been described that are able to be presented at the cell surface independently of protein synthesis and in the absence of Ii and HLA-DM, consistent with their binding to pre-existing molecules within the early endosomes (Nadimi *et al.*, 1991; Pinet *et al.*, 1994, 1995; Lindner & Unanue, 1996; Griffin *et al.*, 1997). Typically, only antigens that do not require extensive processing are suitable for presentation by the recycling receptors (Davidson *et al.*, 1991). The early endosomes exhibit a much reduced catabolic capacity compared with the MIIC (§1.5.6; Schmid *et al.*, 1988), such that only superficial epitopes within a protein are able to be liberated within this environment. Several determinants have been reported to associate with recycling class II MHC molecules as intact, partially unfolded polypeptides (*e.g.* Lindner & Unanue, 1996; Vergelli *et al.*, 1997). The targeting of distinct antigens towards the pool of recycling αβ dimers might be influenced by the individual Ig subunits of the BCR (Bonnerot *et al.*, 1995). Preprocessed peptides may also bind to the class II MHC molecules directly at the cell surface without need of internalisation into the early endosomes (Shimonkevitz *et al.*, 1983; Ceppellini *et al.*, 1989; Busch *et al.*, 1990; Monji & Pious, 1997).

1.6.3 The superantigens

In addition to conventional peptide antigens, class II MHC molecules are also able to present another type of ligand to T lymphocytes, the superantigens (SAgs). These are a

novel class of proteins of microbial origins, so-called because of their potent ability to stimulate high numbers of T cells (an estimated 10–20% of the T cell repertoire compared with a conventional antigen response of <1 in 1000 T cells; reviewed by Li *et al.*, 1999 and Lavoie *et al.*, 1999). Superantigens have been implicated in the development of numerous diseases. The *Staphylococcus aureus* enterotoxins (SEs) are associated with food poisoning (*e.g.* SEA and SEB; Kotzin *et al.*, 1993) and the *S. aureus* TSST-1 superantigen causes toxic shock syndrome (Bohach *et al.*, 1990). The mouse mammary tumour virus (MMTV) superantigens play a critical role in viral transmission (reviewed by Acha-Orbea *et al.*, 1999) and SAgs have also been implicated in the development or progression of rabies (Astoul *et al.*, 1996), autoimmunity (Paliard *et al.*, 1991; Brocke *et al.*, 1993, 1998; Luppi & Trucco, 1996; Renno & Acha-Orbea, 1996; Conrad *et al.*, 1997; Schiffenbauer *et al.*, 1998) and a number of skin conditions (Leung *et al.*, 1993, 1995; Cavarelli *et al.*, 1997; Vath *et al.*, 1997).

The presentation of SAgs by class II MHC molecules is distinct from that of conventional peptide antigens. Processing of the protein by the APC is not required. X-ray crystallographic analyses of a number of different SAg–HLA-DR complexes have revealed that these proteins bind intact, predominantly outside the conventional peptide-binding site (Jardetzky *et al.*, 1994; Kim *et al.*, 1994; Dessen *et al.*, 1997). For example, SEB interacts exclusively with the α_1 domain of the HLA-DR1 heterodimer, to one side of the groove (Jardetzky *et al.*, 1994). TSST-1 associates with both the α_1 and β_1 domains of the class II MHC molecule but also contacts residues of the associated peptide (Kim *et al.*, 1994). The sequence of this peptide may modulate the degree of T cell stimulation by TSST-1 (von Bonin *et al.*, 1995; Wen *et al.*, 1996, 1997).

The interaction between SAgs and T lymphocytes is also unique. The specificity of a T cell for a SAg is determined almost exclusively by the identity of the variable β-chain (Vβ) domain of the TCR (*e.g.* Kappler *et al.*, 1988; MacDonald *et al.*, 1988; Pullen *et al.*, 1988; White *et al.*, 1989; see below, §1.7.1). SAgs take advantage of the organisation inherent in conventional antigen presentation to trigger a subset of peripheral T cells directly through this region (Fields *et al.*, 1996; Li *et al.*, 1998). Such excessive stimulation then leads to a massive release of inflammatory cytokines and the eventual anergy or deletion of the activated T cells (Kawabe & Ochi, 1990, 1991; Rellahan *et al.*, 1990).

1.7. Class II MHC-mediated presentation of antigen to $CD4^+$ T cells

1.7.1 Class II MHC molecules and T cell activation

T lymphocytes interact with MHC molecules on the surface of APC *via* the T cell receptor (TCR). This is a disulphide-linked, transmembrane heterodimer composed of α and β polypeptides (alternatively, a minority of TCR are composed of γ and δ chains; Davis & Bjorkman, 1988). The α and β subunits are members of the Ig superfamily and, like antibodies, are generated clonally by somatic rearrangement of germline variable (*V*), diversity (*D*) and joining (*J*) gene segments. Sequence hypervariability is prominent in the complementarity-determining regions (CDR) that form the binding interface of the TCR with the peptide–MHC complex (For reviews on the structural basis of the recognition of peptide–MHC complexes by T cells, including an examination of recent X-ray crystallographic data, see Garcia & Teyton, 1998; Mazza *et al.*, 1998 and Garcia *et al.*, 1999).

Each TCR participates in a non-covalent interaction with a non-polymorphic, multi-subunit signalling complex, CD3, composed of γ, δ and ε chains and a disulphide-linked ζ–ζ homodimer (Weiss, 1993). The engagement of a peptide-bound MHC molecule by the TCR in the presence of appropriate costimulatory signals and the co-receptor glycoprotein, CD4 or CD8, activates tyrosine kinases leading to the phosphorylation of tyrosine residues within immunoreceptor tyrosine-based activation motifs (ITAMs) in the cytoplasmic tails of the CD3 components. This initiates a cascade of signalling events within the T lymphocytes *via* many different signal transduction pathways (reviewed by Clements *et al.*, 1999; Germain & Stefanova, 1999 and Medema & Borst, 1999).

A T cell becomes activated and mounts an effector response only when a defined number of TCR have been triggered (Viola & Lanzavecchia, 1996; Lanzavecchia, 1997; Lanzavecchia *et al.*, 1999). This threshold may be lowered by the presence of co-stimulatory molecules. The efficiency with which TCR are triggered is influenced by the overall avidity of the MHC–TCR interaction. The avidity is defined as the product of the number of TCR on the cell surface, the number of peptide–MHC complexes upon the APC, the intrinsic affinity of the TCR for this complex and the affinity of the peptide for the MHC molecule (Kim *et al.*, 1996; Alam *et al.*, 1999; Legge *et al.*, 1999). A typical B cell displays on its cell surface 10^5–10^6 class II MHC molecules of any given allotype (Trucco *et al.*, 1980; Ceppellini *et al.*, 1989; Busch *et al.*, 1990; Roucard *et al.*, 1996). The minimum number of specific peptide–class II MHC complexes required for T cell

recognition and proliferation is estimated to be 10–100 *per* cell (Watts & McConnell, 1986; Demotz *et al.*, 1990; Harding & Unanue, 1990; Srinivasan *et al.*, 1991). The sensitivity of this process may lie in the serial engagement of TCR (Valitutti *et al.*, 1995; Valitutti & Lanzavecchia, 1997). The MHC–TCR interaction typically displays a fast rate of dissociation (Corr *et al.*, 1994; Matsui *et al.*, 1994; Sykulev *et al.*, 1994; Lyons *et al.*, 1996). This allows the same peptide–MHC combination to trigger multiple TCR and thus amplify and sustain the activation response, ultimately inducing cell-cycle progression, cytokine production and cellular proliferation.

T cell responses to low-affinity antigens might also be facilitated by the formation of class II MHC superdimers. These complexes contain two parallel MHC molecules oriented in the same direction and have been observed by X-ray crystallography (Brown *et al.*, 1993; Fremont *et al.*, 1996), cell-surface fluorescence imaging (Cherry *et al.*, 1998) and the immunoprecipitation of class II MHC molecules from B cells (Schafer & Pierce, 1994; Roucard *et al.*, 1996; Schafer *et al.*, 1998). It has been suggested that these 'dimers of dimers' may cross-link the TCR and signal B cells to up-regulate co-stimulatory molecules (Brown *et al.*, 1993). The involvement of MHC dimerisation in T cell stimulation is not precluded by the serial engagement model of TCR ligation (Valitutti *et al.*, 1995) and the disruption of contact residues between the class II MHC superdimers has been shown to impact upon the activation of some T cell clones (Goodman *et al.*, 1995; Nydam *et al.*, 1998). However, some evidence for the existence of class II MHC superdimers has been dismissed as crystallisation-packing phenomena and antibody-related artefacts (Hitzel *et al.*, 1999).

1.7.2 MHC molecules and the development of the T cell repertoire

Immunocompetent T cells develop from bone marrow-derived stem cells within the thymus (reviewed by Zuniga-Pflücker & Lenardo, 1996; Rodewald & Fehling, 1998 and Spits *et al.*, 1998). These lymphoid progenitors become committed to the T cell lineage through the actions of cytokines and develop into pro-thymocytes. Proliferation and further differentiation produces the pre-T cells which are now double positive for the co-receptors, CD4 and CD8, and express newly-rearranged αβ TCR and the subunits of the CD3 complex. However, these immature $CD4^+CD8^+$ cells are programmed to die. The rescue of thymocytes from this fate and their final steps of differentiation into mature T cells are dependent upon signals received from interactions between the TCR and MHC molecules.

The presentation of antigen by MHC molecules on the surface of APC is critical to the selection of a functional TCR repertoire from the initial pool of randomly-rearranged αβ dimers. Each clonally-restricted TCR produced by somatic rearrangement of the *V*, *J* and *D* region genes must be assessed for its capacity to interact with MHC molecules of the parental haplotype (*i.e.* MHC restriction). At the same time, the TCR must not exhibit too high an affinity for the self-peptides that are associated with the MHC molecules. This process is of crucial importance in preventing the release of mature, self-reactive T lymphocytes into the periphery, which could lead to the development of autoimmunity.

In common with the activation of T cells in the periphery (§1.7.1), a popular model of T cell selection proposes a defining role for the overall avidity of an MHC molecule for a TCR in determining the fate of an immature T cell, *i.e.* the interrelationship between the number of TCR on the thymocyte, the number of peptide–MHC complexes on the selecting APC, the affinity of the TCR for this complex and the affinity of the peptide for the MHC molecule (Ashton-Rickardt *et al.*, 1994; Ashton-Rickardt & Tonegawa, 1994). Cells that express TCR that are unable to interact productively with the self-MHC, *i.e.* an MHC–TCR interaction of very low avidity, are destined to die from neglect. Those whose TCR bind to peptide–MHC complexes with an overall avidity above this threshold are rescued from programmed cell death by positive selection. However, if the MHC–TCR avidity is too high, negative selection occurs upon this population and the thymocyte is targeted for clonal deletion by apoptosis or functional inactivation (anergy). In this way, T cells are selected to be 'tolerant' of self-antigens (For reviews on T cell selection, see Anderson *et al.*, 1999 and Sebzda *et al.*, 1999)

Double-positive $CD4^+CD8^+$ thymocytes are located predominantly within the thymic cortex (Shortman *et al.*, 1990). Epithelial cells within this site (cTEC) mediate the positive selection of T cells (Anderson *et al.*, 1994; Ernst *et al.*, 1996). The interaction between the TCR and MHC molecules at this stage also defines the lineage commitment of each immature T cell. Thymocytes differentiate into either $CD8^+$ or $CD4^+$ single-positive cells dependent upon the specificity of their TCR αβ dimer for class I or class II MHC molecules on the cTEC (Teh *et al.*, 1988; Kaye *et al.*, 1989). Negative selection of potentially self-reactive T cells takes place subsequently at the cortico-medullary junction upon bone marrow-derived dendritic cells and macrophages (Lo & Sprent, 1986). It is estimated that as few as 3–5% of thymocytes may make the transition from the immature double-positive to mature single-positive phenotype ready for release into the periphery (Shortman *et al.*, 1990).

It is of interest to note that the maturation of class II MHC molecules differs between the two cell populations involved in the thymic selection of $CD4^+$ T cells. While cathepsin S catalyses the late stages of Ii degradation in bone marrow-derived APC (§1.5.1), cathepsin L performs this role in the cTEC (Cresswell, 1998; Nakagawa *et al.*, 1998). Class II MHC molecules expressed by cTEC have also been reported to exhibit a longer association with fragments of Ii than those within medullary TEC or peripheral APC (Farr *et al.*, 1996; Kasai *et al.*, 1996; Oukka *et al.*, 1997). The recent description of a novel serine protease that is restricted in expression to the cTEC provides further evidence of a unique proteolytic environment within these cells (Bowlus *et al.*, 1999). The need for antigen presentation by class II MHC molecules to be equivalent between the APC mediating negative selection in the thymic medulla and those circulating in the periphery is understandable. However, it is less clear why the positively-selecting cTEC should differ.

1.8. A pivotal role for CLIP in the class II MHC pathway of antigen presentation

A remarkable feature of Ii is its ability to maintain chaperone functions for nascent class II MHC molecules even throughout its proteolysis in the endocytic compartments. Essential to this property is the interaction of Ii with the αβ dimers *via* the CLIP region irrespective of allotype, unlike the specificity exhibited for antigen-derived peptides. The generation of the CLIP–αβ complex is a critical point in the class II MHC pathway of antigen presentation. Upon this structure hinges the eventual outcome of every newly-synthesised class II MHC molecule. For example, if CLIP is released from the αβ dimer prematurely, an inappropriate peptide may bind or, equally undesirable, the empty molecules may aggregate and thereby be targeted for degradation. On the other hand, if the timely release of CLIP in the MIIC is disrupted, class II MHC molecules will not duly be able to bind suitable peptide ligands. In both instances, the consequence of this deviation from the desired outcome is the same — deficiency in a critical part of the cell-mediated immune response.

1.9. Project objectives

With the fundamental aim of elucidating the molecular mechanisms underlying the promiscuous associations of CLIP with different class II MHC allotypes, the research presented herein explores the binding of this sequence to the mouse αβ dimers, $I\text{-}A^d$, I-

A^s, I-A^k and I-E^d. At the commencement of this project, the interaction of CLIP with class II MHC had yet to be examined by X-ray crystallography. Nevertheless, there already existed a significant amount of evidence to support the notion that this sequence may occupy the peptide-binding groove, suggesting that CLIP must possess unique features that allow it to overcome the structural variability of each distinct binding groove. It was the immediate goal of this research to determine the basis of this peculiar property of CLIP by examining the binding of side-chain or configurationally-substituted analogues of mouse CLIP86–104 and, additionally, sets of length-altered CLIP and frameshifted 15-mers.

Having defined the general motif by which CLIP interacts with the groove of a variety of class II MHC molecules, it became apparent that this sequence might be employed as a mechanistic probe to compare the binding properties of different allotypes. Of greatest interest would be an investigation to advance our understanding of the relationship between disease susceptibility and certain MHC allelic variants. The non-obese diabetic (NOD) mouse is an animal model of the chronic human autoimmune disorder insulin dependent diabetes mellitus (IDDM) and expresses an atypical class II MHC molecule, I-A^{g7}. This molecule is one of several examples of MHC allotypes known to influence the risk to an individual of developing autoimmunity. The I-A^{g7} molecule contains two unique mutations within the peptide-binding site and therefore may be expected to display altered CLIP binding properties. The differences revealed have been rationalised in terms of the role of I-A^{g7} in diabetes aetiology.

CHAPTER 2.

Materials and Methods

The general experimental protocols and reagents used routinely throughout this research are described in this chapter. All research reported in this thesis was carried out under PC2 laboratory conditions as stipulated by the Genetic Manipulation Advisory Committee (GMAC) of Australia. Use and disposal of radioactive substances was performed in accordance with the Australian National University (ANU) radiation safety guidelines.

2.1. Reagents, buffers and media formulations

The specific chemical reagents used in the laboratory procedures described in this thesis are given in *Appendix A*, together with the supplier's name. Unless specified otherwise, all reagents and chemicals used are of analytical grade. Buffer solutions and cell culture media are detailed in *Appendix B*.

2.2. Cell culture

2.2.1 Cell lines

All antigen-presenting cell lines used in this research are listed in Table 2.1, together with details of the class II MHC molecules expressed and source. Table 2.2 provides information on the class II MHC-restricted T cell hybridomas used in the antigen presentation assays. The mouse helper T cell line, HT-2 (Watson, 1979) used in the interleukin-2 (IL-2) bioassay (§2.7.1) was provided by Prof. H. O. McDevitt, Stanford University, CA, USA.

2.2.2 Cell growth conditions

Mammalian cell lines were maintained in RPMI 1640 culture medium (Commonwealth Serum Laboratories, VIC, Australia; *Appendix B1*) supplemented with 2 g/L sodium bicarbonate, 5–10% (v/v) heat-inactivated foetal bovine serum (FBS; Trace Biosciences, NSW, Australia), 0.05 mM β-mercaptoethanol, 2 mM L-glutamine, 10 mM HEPES and antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin). All media were sterilised by passage through 0.2 µm cellulose acetate membrane bottle-top filters (Costar Scientific, MA, USA) before use. In this thesis, the terms 'culture medium' or 'RPMI' refer to the complete 10% (v/v) FBS/RPMI formulation described here, unless stated otherwise. Cell lines transfected with cDNA encoding class II MHC genes also co-express the *neo*

Table 2.1 *Antigen-presenting cells*

APC	Class II MHC	Source	Reference
A20	$A\alpha^dA\beta^d$, $E\alpha^dE\beta^d$	McDevitt[d]	Kim *et al.* (1979)
CH27	$A\alpha^kA\beta^k$, $E\alpha^kE\beta^k$	McDevitt[d]	Houghton *et al.* (1986)
M12.A3[a]	$E\alpha^dE\beta^d$	Cooke[e]	Glimcher *et al.* (1985)
M12.C3[a]	class II MHC negative	McDevitt[d]	Glimcher *et al.* (1985)
1-5.4[b]	$A\alpha^eA\beta^e$	this lab	Gautam *et al.* (1992)
M12.D[b]	$A\alpha^dA\beta^d$	McDevitt[d]	unpublished
M12.NOD[c]	$E\alpha^dE\beta^d$, $A\alpha^dA\beta^{g7}$	Cooke[e]	Quartey-Papafio *et al.* (1995)
M12.ASP[c]	$E\alpha^dE\beta^d$, $A\alpha^dA\beta^{g7Asp57}$	Cooke[e]	Quartey-Papafio *et al.* (1995)
M12.PRO[c]	$E\alpha^dE\beta^d$, $A\alpha^dA\beta^{g7Pro56}$	Cooke[e]	Quartey-Papafio *et al.* (1995)

[a]Derived by γ-radiation mutagenesis of the H-2^d B cell lymphoma, M12.4.1. The M12.C3 cells express intact $E\alpha^dE\beta^d$ polypeptides but they are glycosylated abnormally and fail to reach the cell surface. In both M12.C3 and M12.A3 cells, the $A\beta^d$ gene is defective but the $A\alpha^d$ gene is transcribed normally and endogenous $A\alpha^d$ may form functional hybrids with transfected Aβ gene products.

[b]Derived from M12.C3 by transfection of cDNA encoding $A\alpha^e$ and $A\beta^e$ (1-5.4) or $A\alpha^d$ and $A\beta^d$ (M12.D).

[c]Derived from M12.A3 by transfection of genomic DNA encoding $A\beta^{g7}$ (M12.NOD) or $A\beta^{g7}$ encoding an aspartic acid at position 57 (M12.ASP) or a proline at position 56 (M12.PRO).

[d]Supplied by Prof. H. O. McDevitt, Stanford University, CA, USA.

[e]Generously provided by Dr A. Cooke, University of Cambridge, Cambridge, UK.

Table 2.2 *T cell hybridomas*

T cell hybridoma	H-2 restriction	Peptide specificity	Source	Reference
3DO-54.8	I-A^d	Ova323–339	Marrack[a]	Shimonkevitz *et al.* (1983)
3A9	I-A^k	HEL46–61	Allen[b]	Allen & Unanue (1984)
1934.4	I-A^u	MBP *Ac*1–11	this lab	Wraith *et al.* (1989)
13.26	I-E^d	SWM132–147	Jones[c]	Morel *et al.* (1987)
12–26λ	I-E^d	λrep12–26	Jones[c]	unpublished
1H11.3	I-E^d	HEL105–120	Adorini[d]	Adorini *et al.* (1988a)
2G7.1	I-E^k	HEL1–18	Adorini[d]	Adorini *et al.* (1991)
4C1.6	I-E^k	HEL108–116	Adorini[d]	Leighton *et al.* (1991)
2B4	I-E^k	mCyt*c*88–103	Davis[e]	Samelson *et al.* (1983)

[a]Gift from Prof. P. Marrack, National Jewish Hospital and Research Centre, CO, USA.
[b]Gift from Prof. P. M. Allen, Washington University School of Medicine, MO, USA.
[c]Gift from Prof. P. P. Jones, Stanford University, CA, USA.
[d]Supplied in collaboration with Dr L. Adorini, Roche Milano Ricerche, Milan, Italy.
[e]Gift from Prof. M. M. Davis, Stanford University, CA, USA.

antibiotic resistance gene and these cultures were positively selected for one week at monthly intervals in culture medium supplemented with the antibiotic, G-418 sulphate (Geneticin; Gibco BRL, NY, USA; Southern & Berg, 1982): 200 μg/mL for 1-5.4 and M12.D; 1 mg/mL for M12.NOD, M12.PRO and M12.ASP. Growth medium for the IL-2-dependent cell line, HT-2, was supplemented with 50 U/mL recombinant mouse IL-2, provided kindly by Ms E. R. O'Neill, JCSMR. All cell lines were treated for one week at 4–5 monthly intervals with Mycoplasma Removal Agent (ICN Biomedicals, Tokyo, Japan) at 0.5 μg/mL.

Cell cultures were maintained in polystyrene, gamma-irradiated tissue culture flasks (Nunc, Roskilda, Denmark) at 37°C in a 5% CO_2 atmosphere within an automated incubator (model 3194; Forma Scientific, OH, USA) with humidity provided by a solution of 0.025% (w/v) benzalkonium chloride/ 1% (w/v) SDS in sterile ddH_2O. Incubators were disinfected regularly with 75% (v/v) ethanol (EtOH) or a solution of the virucidal disinfectant, Virkon (Antec International, Suffolk, UK) prepared according to the manufacturer's specifications.

Centrifugation of cells for the purpose of counting or washing was performed in 15 mL polypropylene, gamma-irradiated, conical Falcon tubes (Becton Dickinson, NJ, USA) at 300 × *g* for 3–4 minutes in a Clements 2000S benchtop centrifuge (1200 rpm; NSW, Australia). Viable cell numbers were determined by the exclusion of the vital dye, trypan blue (0.4% w/v; Sigma Chemical Co., MO, USA), as observed on a haemocytometer under a light microscope.

2.2.3 Cryogenic storage of cell culture collections

Samples of all cell lines used in this research were stored cryogenically to maintain tissue culture collections. Healthy, rapidly-dividing cells were pelleted by centrifugation at 4°C (300 × *g*, 3–4 min) and washed once in fresh RPMI. Cell pellets were resuspended at 10^6 cells *per* mL in chilled culture medium containing 10% (v/v) dimethyl sulphoxide (DMSO) as a cryoprotectant. Aliquots of 1 mL were dispensed into polypropylene CryoTubes (Nunc, Roskilda, Denmark) and frozen slowly on dry ice before transferring to liquid nitrogen for long-term preservation.

To reconstitute frozen cell lines, vials were thawed quickly in a 37°C waterbath and 9 mL of culture medium (prewarmed to 37°C) was added immediately to dilute the DMSO

cryoprotectant. Cells were washed twice in fresh RPMI to remove any traces of DMSO and resuspended in a sterile culture vessel for cell proliferation.

2.3. Synthetic peptides

2.3.1 Peptide synthesis

The synthetic peptides used in this research are listed in Table 2.3. Specific substituted and/or truncated analogues of CLIP are detailed in the relevant chapters. All peptide syntheses were performed at the ANU Biomolecular Resource Facility (ACT, Australia) by Mr K. N. McAndrew and Dr P. J. Milburn.

The sets of substituted CLIP α-carboxamide analogues were assembled using the solid-phase simultaneous multiple-peptide synthesis (SMPS) methodology described by Houghten (1985) which employs standard *tert*-butoxycarbonyl (*t*-Boc) chemistry and 1% cross-linked *p*-methylbenzhydrylamine polystyrene resin. Following α-deprotection, side-chain deprotection and cleavage from the resin were achieved by solvolysis in liquid hydrogen fluoride using 10% anisole as a carbonium ion scavenger (Houghten *et al.*, 1986). The peptide and resin were then washed twice with diethyl ether to remove organic compounds and the peptide was dissolved in 10% acetic acid and lyophilised.

Antigen-derived peptides and large batches of CLIP were synthesised using standard 9-fluorenylmethoxycarbonyl (Fmoc)/*N*-methylpyrolidone chemistry on 1% cross-linked *p*-hydroxymethylphenoxymethylpolystyrene resin with an Applied Biosystems peptide synthesiser (model 430A; Perkin Elmer, CA, USA). Cleavage and deprotection were achieved in 95% TFA in the presence of the appropriate scavengers. Crude peptides synthesised by either Fmoc or *t*-Boc chemistry were purified to premium grade (>99% purity) by dissolving in a minimal volume of 3 M guanidine thiocyanate, 0.1 M potassium phosphate, pH 4.0 and eluting from reversed-phase C18 HPLC columns using acetonitrile gradients in 0.1% aqueous trifluoroacetic acid (TFA). Product integrity was confirmed by mass spectroscopy and lyophilised peptide stocks were stored in chemically-resistant, air-tight plastic vials at -20°C. To reduce moisture absorption, vessels were equilibrated to room temperature before opening.

Where required, biotinyl moieties were conjugated to peptides while still on the resin. *N*-terminal biotinylation was performed after α-deprotection in a solution of 20 mM sulfosuccinimidyl 6-(biotinamido) hexanoate (Pierce, IL, USA; 1.0 mL *per* 20 μmoles of

Table 2.3 *Synthetic peptides*

Source protein	Peptide	Amino acid sequence	MW	H-2 restriction[d]
Bacteriophage λ cI repressor	λrep12–26	LEDARRLKAIYEKKK	1862	I-A^{k}, I-E^{d,k}
Heat shock protein, mouse (HSP60)	p12(166–185)	EEIAQVATISANGDKDIGNI	2059	I-A^{g7}
Hen egg lysozyme	HEL1–18	KVFGRCELAAAMKRHGLD	2003	I-E^{k}
	HEL10–23	AAMKRHGLDNYRGY	1652	I-A^{g7}
	HEL46–61	NTDGSTDYGILQINSR	1754	I-A^{d,k}
	HEL105–120	MNAWVAWRNRCKGTDV	1907	I-E^{d,k}, I-A^{k}
Invariant chain, mouse	CLIP81–109[a]	LPKSAKPVSQMRMATPLLMRPMSMDNMLL	3291	all mouse allotypes
Moth cytochrome *c*	mCyt*c*88–103	ANERADLIAYLKQATK	1805	I-E^{k}
Mouse serum albumin	MSA560–574	KPKATAEQLKTVMDD	1675	I-A^{g7}
Myelin basic protein, rat	MBP *Ac*1–11[b]	*Ac*ASQKRPSQRHG	1293	I-A^{u}
Myelin oligodendrocyte glycoprotein, rat	rMOG8–22	PGYPIRALVGDEQED	1659	I-A^{g7}
Ovalbumin, chicken egg	Ova322–339[c]	KISQAVHAAHAEINEAGR	1902	I-A^{d,k,u,g7}
Sperm whale myoglobin	SWM132–147	NKALELFRKDIAAKYK	1909	I-E^{d}

[a]Mouse CLIP is numbered according to the human p31 Ii sequence (*Chapter 1*, Figure 1.5) throughout this work for ease of comparison between these systems.
[b]*Ac* denotes amino-terminal acetylation
[c]Unless to be biotinylated, ovalbumin peptide was synthesised with an additional C-terminal tyrosine residue (Ova323–339Y) to increase solubility in aqueous solutions.
[d]I-A^{d,k}-binding peptides taken from Sette *et al.* (1989a) and Adorini *et al.* (1988b); I-E^{d,k}-binding peptides from Schild *et al.* (1995); I-A^{u}-binding peptides from Zamvil *et al.*, 1986 (MBP *Ac*1–11) and Gautam *et al.*, 1992 (Ova323–339); I-A^{g7}-binding peptides from Bockova *et al.*, 1997 (p12(166–185)), Hurtenbach *et al.*, 1993 (HEL10–23), Reich *et al.*, 1994 (MSA560–574), Amor *et al.*, 1996 (rMOG8–22) and Smilek *et al.*, 1990 (Ova323–339).

peptide) and 100 mM *N*-hydroxybenzotriazole in 1:1 (v/v) *N,N*-dimethylformamide/dichloromethane. The reaction mixture was shaken vigorously at room temperature for 1 hour. The biotinylated peptides were deprotected, cleaved and purified following normal procedure.

2.3.2 Preparation of peptide stock solutions

Lyophilised peptides were reconstituted to a stock concentration of 5 mM in sterile phosphate-buffered saline (PBS; Appendix B1) containing 0.01% (w/v) sodium azide. To help preserve the chemical integrity of peptides in solution, stocks were stored at -20°C in single-use aliquots. Peptides that are poorly soluble in aqueous solutions may exhibit cytopathic effects on lymphocytes at high concentrations *in vitro* (Amor *et al.*, 1996). To aid dissolution of such peptides, a small volume of sterile 20% (v/v) DMSO/PBS was added. For antigen presentation assays and cell-surface peptide binding assays, peptides were diluted subsequently into RPMI culture medium, thereby avoiding possible cytotoxic effects by ensuring a final concentration of DMSO of less than 0.1%. For assays using purified class II MHC, known water-insoluble peptides were dissolved at 10 mM in 100% DMSO and diluted subsequently to 1–5 mM with aqueous 0.01% (w/v) NaN_3/PBS solution.

2.3.3 Measurement of circular dichroism

To examine the secondary structure of D-amino acid-substituted CLIP86–104, circular dichroic (CD) spectra were measured. Peptides were dissolved in 50% (v/v) trifluoroethanol/ 10 mM sodium phosphate, pH 7.4 at a concentration of 50 µM and placed into a quartz cell with a 1 mm pathlength. Circular dichroism was recorded at 25°C using a Jovin Yvon CD6 spectrometer (Instruments SA, NJ, USA) with time averaging over 2 seconds *per* 0.2 nm increment. All CD measurements were performed by Dr P. J. Milburn of the ANU Biomolecular Resource Facility, ACT, Australia.

2.4. Antibodies

2.4.1 Antibody sources

All monoclonal antibodies used in this research are listed in Table 2.4, together with their

Table 2.4 *Monoclonal antibodies.*

Antibody	Specificity	Subclass	Hybridoma	Source	Reference
14-4-4S	I-E$\alpha^{d,k,p,r,u}$	IgG_{2a}	ATCC HB 32	McDevitt[d]	Ozato *et al.* (1980)
MK-D6	I-A$\beta^{d,p,q}$	IgG_{2a}	ATCC HB 3	McDevitt[d]	Kappler *et al.* (1981)
10-3.6.2	I-A$\beta^{f,k,r,s,u,g7}$	IgG_{2a}	ATCC TIB 92	McDevitt[d]	Oi *et al.* (1978)
10-2.16	I-A$\beta^{f,k,r,s,u,g7}$	IgG_{2b}	ATCC TIB 93	ATCC[e]	Oi *et al.* (1978)
M5/114.15.2	I-A$\beta^{b,d,q}$, I-E^{d,k}	IgG_{2b} (rat)	ATCC TIB 120	McDevitt[d]	Bhattacharya *et al.* (1981)
OX6[a]	I-A^{f,k,r,s,u,g7}	IgG_1	MRC-OX6	Cooke[f]	McMaster & Williams (1979)
P4H5	Ii lumenal domain[b]	IgG_1	P4H5	Jones[g]	Mehringer *et al.* (1991)
In-1	Ii cytoplasmic domain[c]	IgG_{2b}	In-1	Jones[g]	Koch *et al.* (1982)

[a]OX6 recognises a common determinant present on H-2A molecules of several mouse strains including NOD (I-A^{g7}) and the NOD mutants I-A^{g7Asp57} and I-A^{g7Pro56} (Quartey-Papafio *et al.*, 1995) but not I-A^{d} (Liu *et al.*, 1993). It was raised against rat I-A-equivalent, RT1.B class II MHC molecules.
[b]Hamster mAb raised against the mouse Ii peptide 100–117 (RPMSMDNMLLGPVKNVTK)
[c]Recognises a determinant in the N-terminal cytoplasmic tail of Ii.
[d]Supplied by Prof. H. O. McDevitt, Stanford University, CA, USA.
[e]Purchased from the American Type Culture Collection, MD, USA.
[f]Purified OX6-FITC was kindly provided by Dr A. Cooke, University of Cambridge, Cambridge, UK.
[g]Gift from Prof. P. P. Jones, Stanford University, CA, USA.

specificity, IgG subclass and source.

The following polyclonal antibodies were purchased from Pierce (IL, USA):
- Goat anti-mouse IgG (H+L), unconjugated
- Goat anti-rat IgG (H+L), unconjugated
- Goat anti-rat IgG (H+L), horseradish peroxidase-conjugated

2.4.2 Protein A purification of monoclonal antibodies

Monoclonal antibodies were purified by the low salt, Protein A–Sepharose affinity-chromatography method described by Harlow & Lane (1988). Ascitic fluid or the culture supernatant of mAb-producing hybridomas were used as sources of crude antibody, estimated to contain between 1–10 mg/mL and 20–50 µg/mL of antibody, respectively (Harlow & Lane, 1988). Briefly, 1–2 mL columns of pre-swollen Protein A–Sepharose 6 MB (Pharmacia Biotech, Uppsala, Sweden) were poured and equilibrated in 50 mL 100 mM Tris, pH 8.0. A 1 mL column of swollen Protein A–Sepharose may adsorb between 10–20 mg of pure IgG (Harlow & Lane, 1988) and is therefore sufficient for 1–2 mL ascitic fluid or 200–400 mL of culture supernatant. The pH of the antibody solution was adjusted to 8.0 with a 1/10 volume of 1.0 M Tris, pH 8.0 and the solution was passed several times through the protein A–Sepharose column. The beads were washed sequentially with 10 column volumes each of 100 mM Tris, pH 8.0 and 10 mM Tris, pH 8.0 (*Appendix B2.1*). Antibody was eluted with 100 mM glycine, pH 3.0 and 15 samples of 500 µL each were collected into 50 µL 1 M Tris, pH 8.0 in microfuge tubes. Fractions containing the immunoglobulins were identified from UV absorbance readings at 280 nm and the concentration of the purified mAb was calculated (A_{280} of 1 = 0.75 mg/mL IgG; Harlow & Lane, 1988). All antibody-containing fractions were pooled and dialysed (cellulose acetate tubing, MW cutoff ≥ 12000; Sigma Chemical Co., MO, USA) against PBS for 2 days at 4°C. Purified antibody was stored at 4°C in PBS with NaN_3 added to 0.02% (w/v).

2.4.3 Biotinylation of monoclonal antibodies

Monoclonal antibodies were conjugated to an *N*-hydroxysuccinimido ester of biotin using the method of Goding (1983). Briefly, 1–2 mg/mL purified monoclonal antibody was dialysed at 4°C against 500 mL biotin labelling buffer, pH 8.4 (*Appendix B2.2*) with three changes over 2 days. The protein concentration was determined by the UV

absorbance at 280 nm (A_{280}). A fresh 10 mg/mL anhydrous solution of *N*-hydroxysuccinimidobiotin (NHS-biotin; Pierce, IL, USA) was prepared in DMSO and 10 µL was added for each milligram of antibody. The conjugation mixture was incubated for 1–2 h at room temperature then unreacted biotinylation reagent was removed by dialysis at 4°C against 500 mL 0.1% (w/v) NaN_3/PBS with three changes over 2 days. Biotinylated mAb were stored at 4°C.

2.4.4 Fluoresceination of monoclonal antibodies

Monoclonal antibodies were conjugated to the fluorochrome, fluorescein isothiocyanate (FITC) using the method described by Coligan *et al.* (1994). Briefly, purified monoclonal antibody was dialysed against 500 mL FITC labelling buffer (*Appendix B2.3*) at 4°C with three changes over 2 days to remove free NH_4^+ ions and raise the pH to 9.2. The concentration of the dialysed antibody was determined from the UV absorbance at 280 nm. A 5 mg/mL stock of anhydrous FITC (Pierce, IL, USA) in DMSO was prepared immediately before use and 20 µL was added dropwise for each milligram of antibody. The tube containing the conjugation mixture was wrapped in aluminium foil and agitated by inversion for 2 hours at room temperature. Unreacted FITC was removed by dialysis in 500 mL FITC dialysis buffer (*Appendix B2.3*) at 4°C in darkness with three changes over 2 days. Fluoresceinated mAb were stored in darkness at 4°C.

2.5. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and protein detection

Proteins were separated for analytical purposes using SDS-PAGE. All gel electrophoresis was performed using an XCell II Mini-Cell apparatus (Novel Experimental Technology (NOVEX), CA, USA).

2.5.1 Tris-glycine SDS-PAGE

SDS-PAGE was performed routinely using the Tris-glycine gel system (Laemmli, 1970). All Tris-glycine gels were prepared according to the formulations given by Harlow & Lane (1988). Protein samples were diluted with Tris-glycine sample buffer containing 2% (w/v) SDS (*Appendix B3.1*) and loaded onto a 5% stacking gel (pH 6.8) overlaying a resolving gel (pH 8.8) of 10%, 12% or 15% (w/v) acrylamide (*Appendix B3.1*).

Denatured samples were prepared in sample buffer supplemented with 2–5% (v/v) of the reducing agent, 2-ME, and boiled for 5 min before loading. In accordance with a discontinuous buffer system, gels were run in a Tris-glycine running buffer, pH 8.3 (*Appendix B3.1*) and all buffers contained 0.1% (w/v) SDS. Electrophoresis was performed at a constant voltage of 80 V through the stacking gel and then increased to 150 V for protein resolution. Molecular weight ranges were visualised during electrophoresis using rainbow-coloured high-range protein MW markers (Amersham, Buckinghamshire, UK) prepared for loading as for the denatured samples.

2.5.2 Tris-tricine SDS-PAGE

For the resolution of low molecular weight proteins and large peptides, samples were separated on pre-cast NOVEX Tris-tricine gradient gels (10–20% (w/v) acrylamide) using a discontinuous buffer system, as developed by Schägger & von Jagow (1987). Samples were prepared for loading by dilution in Tris-tricine sample buffer containing 2% (w/v) SDS (*Appendix B3.1*). For denatured samples, the sample buffer was supplemented with 2–5% (v/v) 2-ME and samples were boiled for 5 minutes before loading. Gel running buffers consisted of 0.2 M Tris, pH 8.9 in the outer tank (anode; *Appendix B3.1*) and a Tris-tricine buffer, pH 8.3 containing 0.1% SDS in the inner tank (cathode; *Appendix B3.1*). Gels were run at 125 V constant voltage for 90 min. As with Tris-glycine gels (§2.5.1), the inclusion of a rainbow MW marker on each gel permitted visualisation of electrophoresis.

2.5.3 Silver staining of SDS-polyacrylamide protein gels

Proteins were stained with silver salts using the method described in detail by Sambrook *et al.* (1989). Protein samples were separated on 12% SDS-polyacrylamide gels by the standard protocol (§2.5.1). To estimate the molecular weight of electrophoresed proteins, a 2 µL volume of uncoloured, mid-range protein molecular weight markers (Promega, WI, USA) was run alongside the protein samples on each gel prepared for silver staining.

Upon completion of SDS-PAGE, gels were removed from their plates and rinsed briefly in ddH_2O. To obtain optimal silver staining results, handling of gels was minimised and all silver staining procedures were performed using scrupulously clean glassware. All solutions were prepared using ddH_2O to minimise contaminants that may interfere with the sensitivity of silver staining. Electrophoresed proteins were fixed in gel-fixing

solution (*Appendix B3.2*) for 3–12 h at room temperature. An extended fixing time was found to be optimal to ensure complete diffusion of SDS and 2-ME from the gel matrix which interfered with silver staining. When the acid had leeched fully into the gel (bromophenol blue in the sample buffer turns yellow), gels were rocked gently in 30% (v/v) EtOH for 30 minutes twice, then rehydrated with three 10 min washes in ddH_2O. Proteins were stained using a 0.1% (w/v) solution of silver nitrate (freshly diluted in ddH_2O from a 20% (w/v) stock; *Appendix B3.2*) for 30 min. Both sides of the gel were washed for 20 seconds under a gentle stream of ddH_2O, then the stain was developed by incubating the gel in a freshly-prepared solution of sodium carbonate/ formaldehyde (*Appendix B3.2*) until bands were visible. The reaction was quenched in a 1% (v/v) acetic acid solution for 5 min and the gel was washed twice in ddH_2O for 10–20 min. Gels were dried at 80°C for 2 h onto damp blotting paper using a vacuum gel drier (model 583; Bio-Rad, CA, USA).

2.5.4 Western blotting

For immunodetection and analysis, proteins were immobilised on Trans-Blot nitrocellulose membranes (Bio-Rad, CA, USA) by electrophoretic transfer from SDS-polyacrylamide gels. Electroblotting was performed in an XCell II Mini-Cell Blot module (NOVEX, CA, USA) at 30 V constant for 1.5–2 h using the NOVEX-recommended Western transfer buffer (*Appendix B3.3*). The efficiency of transfer was monitored by the movement of pre-stained rainbow protein standards (Amersham, Buckinghamshire, UK).

After transfer, nitrocellulose membranes were washed briefly with PBS and then dried at room temperature for at least 30 minutes. To prevent non-specific adsorption of immunoreagents, membranes were incubated in Western blocking buffer containing 0.1% (v/v) Tween-20 and 5% (w/v) low fat milk powder (*Appendix B3.3*) either at room temperature for 1.5 h or at 4°C overnight. Biotinylated primary antibody was diluted as appropriate in Western blocking buffer and bound to target proteins on the membrane by incubation either overnight at 4°C or for 1.5 h at room temperature. Unbound primary antibody was removed by rinsing membranes twice briefly in Western wash buffer containing 0.1% (v/v) Tween-20 (PBS–T; *Appendix B3.3*) followed by 3 longer washes (1×15 min, 2×5 min). Membranes were probed with a horseradish peroxidase-streptavidin conjugate (S–HRP; Pierce, IL, USA) for 1 h at room temperature followed by three washes in Western blocking buffer (15 min each) and two washes in Western

wash buffer (5 min each). Protein immunodetection was achieved by enhanced chemiluminescence (ECL) using a Western blot ECL kit (Amersham, Buckinghamshire, UK). Chemiluminescent blots were recorded on X-OMAT autoradiography film (Kodak, NY, USA) and the molecular weight of proteins estimated by reference to ECL biotinylated protein molecular weight standards (Amersham, Buckinghamshire, UK).

2.6. Purification of class II MHC proteins

Mouse class II MHC molecules were purified from detergent lysates of B cell lymphomas by immunoaffinity chromatography using a method adapted from Gorga *et al.* (1987).

2.6.1 Preparation of antibody affinity columns

The Sepharose column matrix was prepared for coupling according to the manufacturer's recommendations. Briefly, 1 g of CNBr-activated Sepharose 4B powder (Pharmacia Biotech, Uppsala, Sweden) was swollen in 20 mL 1 mM HCl for 2 h then washed in a sintered glass funnel with a further 300 mL 1 mM HCl to remove freeze-drying additives. The Sepharose beads were rinsed in 30 mL coupling buffer, pH 8.3 (*Appendix B4.1*) and the pH checked to be 8–9. Purified class II MHC-specific mAb (10 mg *per* column) was dialysed against 1 L coupling buffer overnight at 4°C and the A_{280} of the dialysed mAb was recorded. A thick slurry of resin was added to the mAb and rocked either for 2 h at room temperature or overnight at 4°C. The resin was pelleted at $50 \times g$ for 1 min using a Clements 2000S benchtop centrifuge (500 rpm; NSW, Australia) and the A_{280} of the supernatant measured again to confirm antibody conjugation to the Sepharose matrix. To block any remaining active groups, the beads were resuspended in 5 mL 0.2 M glycine, pH 8.0 for either 3 h at room temperature or overnight at 4°C. Antibody-coupled beads were then washed sequentially in 50 mL coupling buffer, 50 mL 0.1 M acetate buffer (*Appendix B4.1*) and 50 mL 0.5% (v/v) IGEPAL/PBS, centrifuging between washes at $50 \times g$ for 2 min. Columns were stored at 4°C in 0.05% (w/v) NaN_3/ 0.5% (v/v) IGEPAL/PBS.

2.6.2 Affinity purification of class II MHC molecules

Class II MHC-expressing cells were grown in bulk in sterile roller bottles in 5% FBS/ RPMI to a minimum of 10^9 cells total. The cells were pelleted at $540 \times g$ for 30 min at

4°C in 1 L polypropylene bottles using a Beckman J-6M/E centrifuge (rotor JS-4.2, 1500 rpm; CA, USA) and resuspended in a small volume of ice-cold PBS. Cell suspensions were transferred to 50 mL polypropylene Falcon tubes (Becton Dickinson, NJ, USA) and washed twice more with PBS, pelleting cells between washes at 300 × *g* for 5 min at 4°C (Jouan CR412 centrifuge, 1200 rpm; St Nazaire, France). To lyse membranes, cells were incubated for 10 min on ice at 10^8 cells/mL in PBS lysis buffer containing 0.5% (v/v) of the non-ionic detergent, IGEPAL CA-630 (Sigma Chemical Co., MO, USA) and a cocktail of protease inhibitors (*Appendix B4.2*). The lysate was centrifuged at 1600 × *g* at 4°C for 30 min (Jouan CR412, 3000 rpm; St Nazaire, France) to remove large cellular debris and nuclear material and the resulting supernatant was cleared of cytoskeletal elements by ultracentrifugation in polyallomer Quick-Seal tubes (Beckman, CA, USA) at 50 000 × *g* for 1 h in a Beckman L8-70 ultracentrifuge (rotor 70.1Ti, 23 500 rpm; CA, USA) precooled to 4°C.

For immunoaffinity purification, the detergent lysate was passed through a 0.2 µm cellulose acetate Minisart syringe filter (Sartorius, Göttingen, Germany) then the mixture applied three times to a prepared mAb column. The column was washed with 200 mL 0.5% (v/v) IGEPAL/ PBS before the proteins were eluted with class II MHC elution buffer, pH 11.5 (*Appendix B4.2*). Approximately fifteen 1.5 mL fractions of eluate were collected into microfuge tubes, each containing 120 µL citrate–phosphate buffer, pH 5.0 (*Appendix B4.2*) for immediate neutralisation. Following each class II MHC preparation, columns were washed with a further 5 mL elution buffer then 100 mL 0.5% (v/v) IGEPAL/PBS and stored as before.

Samples containing the class II MHC proteins were identified by silver staining on a 12% SDS-polyacrylamide gel and confirmed by Western blotting. Such fractions were pooled and dialysed (cellulose acetate tubing, MW cutoff ≥ 12000; Sigma Chemical Co., MO, USA) for 2 days at 4°C against 0.5% (v/v) IGEPAL/ PBS containing 0.025% (w/v) NaN_3 (*Appendix B4.2*). Purified class II MHC molecules were then spin-concentrated at 5000 × *g* in a Sorvall RC-5B centrifuge (rotor SS-34, 6500 rpm; Du Pont, CT, USA) at room temperature using Centricon-10 ultrafiltration microconcentrators (Amicon, MA, USA) and stored at 4°C.

2.7. Peptide binding assays

2.7.1 Antigen presentation assay

The formation of specific peptide–class II MHC complexes was assessed by quantifying T cell activation using an IL-2 bioassay, as described by Coligan *et al.* (1994).

Serial dilutions of peptide antigen in 50 μL RPMI (concentration range typically 100–1.5 μM) were prepared in triplicate wells of a polystyrene 96-well flat-bottom microtitre plate (Nunc, Roskilde, Denmark). T cell hybridoma and APC cultures were washed twice in normal medium and resuspended at 5×10^5 cells/mL. To each well was added 50 μL of each cell suspension (2.5×10^4 cells *per* well) plus 50 μL normal medium to give a total volume of 200 μL *per* well. To measure background, 100 μL of media was added to 100 μL APC/ T cell hybridoma suspension with no antigen. Plates were incubated at 37°C for 18–24 h, then 150 μL/well culture supernatant was harvested into a fresh 96-well microtitre plate and frozen for 30 min at -70°C to lyse any suspended cells.

To measure T cell responses, the culture supernatant was assayed for IL-2 activity using a secondary culture of the IL-2-dependent T cell line, HT-2 (Watson, 1979). HT-2 cells were prepared first by washing 4× in fresh normal medium to remove residual IL-2, then resuspended at 10^5 cells/mL and 50 μL/well added to an equal volume of thawed culture supernatant. A positive control of 50 μL HT-2 cells growing in 50 μL normal medium supplemented with 50 U/mL recombinant mouse IL-2 was established. HT-2 cells were cultured in the supernatant overnight and the degree of proliferation determined by a 4 hour pulse at 37°C with 0.5 μCi/well [^{3}H]thymidine (Amersham, NSW, Australia; *Appendix B5.1*). Cells were harvested onto glass fibre filters using a Micro Cell Harvester (Skatron Instruments AS, Lier, Norway) and the filter radioactivity counted on a Betaplate liquid scintillation counter (model 1205; Wallac Oy, Turku, Finland). [^{3}H]thymidine incorporation data was recorded as counts *per* minute (cpm).

For competition assays, APC and T cell hybridomas were incubated in triplicate wells with a 50 μL serial dilution of the competitor peptide (concentration range typically 200–3 μM) and a single dose of antigenic peptide chosen from a preliminary titration assay. The maximal level of T cell activation was shown in triplicate wells containing antigen but no competitor. Each competitor was tested in at least two independent experiments.

2.7.2 Cell-surface peptide binding assay

The ability of specific peptides to bind to cell-surface class II MHC molecules was examined by flow cytometry. Using a modification of the method developed by Busch *et al.* (1990), cells were incubated with synthetic peptides conjugated to biotin and then stained with an avidin–FITC conjugate (Pierce, IL, USA). Analysis of stained cells by flow cytometry yields a fluorescent signal related to the number of biotinylated peptide–MHC complexes formed.

2.7.2.1 Flow cytometry system

Flow cytometry experiments were performed on a Becton Dickinson FACScan flow cytometer (CA, USA) using Hewlett Packard Lysis II software for data acquisition. Post-acquisition analysis was performed using the WinMDI software package written and kindly provided by J. Trotter of the Scripps Research Institute, CA, USA.

For each cell sample, the forward light scatter (FSC detector), side light scatter (SSC detector) and FITC fluorescence (FL1 detector, 530 ± 30 nm bandpass filter) of 10 000 events were collected. Only viable cells were measured, as determined by electronic gating on FL3 (670 nm longpass filter) of cells staining positive for the DNA-binding fluorochrome, propidium iodide (PI; Sigma Chemical Co., MO, USA). Cellular debris were eliminated from consideration by gating on the FSC *versus* SSC dot plot during post-acquisition analysis. In all experiments, data were collected at 256 channel resolution and cell-surface fluorescence signals were expressed as the median fluorescence intensity (MFI) to represent accurately the central tendency of the cell population.

2.7.2.2 Preparation of cell samples for flow cytometric analysis

All reagents used in the flow cytometry experiments were diluted into a PBS-based FACS medium (*Appendix B5.2*) containing 1% (w/v) bovine serum albumin (BSA, Fraction V; Armour Pharmaceuticals, Eastbourne, UK) or 1% (v/v) heat-inactivated FBS.

To analyse the binding of a biotinylated peptide to a particular MHC allotype, a dilution series of the peptide (concentration range, 250–8 μM) was prepared in a polystyrene 96-

well round-bottom microtitre plate (Corning, NY, USA). APC were washed once in fresh RPMI, resuspended at 2×10^6 cells/mL and a 50 µL volume was dispensed into each sample well, *i.e.* 100 000 live cells *per* sample at the time of establishing the experiment. All subsequent incubations and washes were performed within the microtitre plate unless otherwise stated. Centrifugations were conducted at 4°C for 3–4 min at 300 $\times g$ in a Jouan CR412 centrifuge (1200 rpm; St Nazaire, France).

Cells were incubated for 18–20 h at 37°C in a CO_2 incubator to allow for peptide binding. Excess unbound peptide was removed by washing cells twice in 200 µL ice-cold FACS medium at 4°C and all subsequent steps were performed on ice to keep cellular metabolic activities at a minimum. To detect biotinyl groups bound on the plasma membrane, cells were stained with 100 µL avidin-FITC for 30 minutes. Total cell-surface class II MHC expression was quantified for each experiment by staining a control cell sample with an appropriate FITC-conjugated mAb. After incubation with fluorochrome-conjugated species, samples were washed three times (twice with 200 µL ice-cold FACS medium, once with 100 µL PI (typically 10 µg/mL; *Appendix B5.2*)), resuspended in 150 µL ice-cold FACS medium and transferred into individual 1 mL round-bottom, polystyrene tubes (50 × 6 mm; Bacto, NSW, Australia) for flow cytometry sample analysis.

For competitive cell-surface peptide binding assays, APC were coincubated for 18–20 hours with a dilution series of unbiotinylated competitor (250–16 µM) and a single dose of biotinylated peptide selected from an initial titration assay. Samples were prepared and analysed as described above. Each competitor was tested in at least two independent experiments and the mean dose yielding 50% inhibition (IC_{50}) was calculated by non-linear regression using the data analysis software package, GraphPad Prism (Version 2.0; GraphPad Software, Inc., CA, USA). Briefly, competitive binding curves were fit to the data using the built-in sigmoidal dose-response (variable slope) equation. The top plateau of the curve was set as the maximum signal of the biotinylated peptide binding alone, obtained experimentally from a duplicate sample containing no competitor. The bottom plateau was set as zero following subtraction of the signal arising from non-specific associations, as measured in the absence of the biotinylated peptide.

Prior to their use in peptide-binding flow cytometry experiments, a suitable working concentration of all dye-conjugated mAb (biotinylated or fluoresceinated) was determined by titrating them against a suitable cell line. Each new batch of avidin-FITC was titrated against the A20 cell line pre-incubated with a working concentration of the biotinylated mAb, MK-D6.

2.7.3 Purified class II MHC binding assay

The binding of biotinylated peptides to immunoaffinity-purified class II MHC molecules was measured using an adaptation of the method of Jensen (1991).

2.7.3.1 Microtitre plate preparation

Polystyrene 96-well IMMULON microtitre plates (Dynex Technologies, VA, USA) were prepared with an initial coating of 100 µL/well of 50 µg/mL goat anti-mouse IgG (H+L) (Pierce, IL, USA; *Appendix B5.3*) in PBS overnight at 4°C. Plates were washed thoroughly with 0.1% (v/v) Tween-20/PBS (PBS–T) three times and 100 µL/well of anti-class II MHC antibody was added at 50 µg/mL in borate buffer, pH 8.0 (*Appendix B5.3*). Plates were incubated overnight at 4°C then washed in PBS–T as before. To block non-specific binding sites, plates were incubated with 100 µL/well milk buffer (*Appendix B5.3*) overnight at 4°C, then washed as before and stored at 4°C with 100 µL/well of 0.01% (w/v) azide/PBS–T.

2.7.3.2 Formation of peptide–class II MHC complexes

In a polystyrene, 96-well, round-bottom microtitre plate (Corning, NY, USA), 25 µL of immunoaffinity-purified class II MHC was added to an equal volume of a biotinylated peptide dilution series (concentration range typically 100–1 µM) in a citrate–phosphate binding buffer, pH 5.0 (*Appendix B5.3*). To determine levels of non-specific binding, negative controls were established consisting of MHC alone, peptide alone and binding buffer with neither MHC nor peptide. The plates were sealed with parafilm to prevent evaporation and the reaction mixture was incubated at 37°C for 24–48 hours.

2.7.3.3 Class II MHC capture and ELISA plate development

To prepare antibody-coated ELISA plates for MHC capture, the plates were washed three times in PBS–T and 100 µL MBN solution was added to each well (*Appendix B5.3*). The peptide–MHC binding mixture was neutralised for capture with 50 µL/well of Tris neutralising buffer (*Appendix B5.3*), then the MBN was discarded from the ELISA plate and 100 µL of the neutralised solution was added. Plates were incubated for 3 h at RT to

allow for MHC capture, then washed three times in PBS–T and 100 µL/well of 1/1000 streptavidin–HRP (Pierce, IL, USA) in PBS–T was added. Plates were incubated for 1 h at RT, washed four times in PBS–T and 100 µL/well of ABTS/ H_2O_2 substrate mixture (*Appendix B5.3*) was added. The presence of bound biotinylated peptide–class II MHC complexes was determined by the development of a green colour after incubating the plate in darkness at room temperature for at least 30 minutes. ELISA plates were read at 405 nm with a reference wavelength of 495 nm on a THERMOmax microplate reader (Molecular Devices, CA, USA) and measurements were recorded using the SOFTmax software package provided by the manufacturer. Data are presented as absorbance readings at 405 nm in optical density (OD) units.

For competition studies, each new preparation of purified class II MHC was first titrated in a binding assay against a biotinylated peptide dilution series to determine the best concentration combination. In each competition experiment, 12.5 µL purified MHC was incubated with 25 µL of a dose range of non-labelled competitor peptide (dilutions 100–12.5 µM) and 12.5 µL of a single dose of biotinylated peptide was selected from the preliminary titration assay. All competitor peptides were tested in at least two independent assays. IC_{50} values were calculated as described previously (§2.7.2).

CHAPTER 3.

The contribution of individual CLIP side chains to the interaction with mouse I-A class II MHC molecules

3.1. Introduction

The particular array of peptide antigens that may be presented by a given class II MHC molecule to $CD4^+$ T lymphocytes is dictated by the amino acid composition of the peptide-binding groove. Specifically, the residues of the α_1 and β_1 domains which fold to form the pockets within this site may vary by up to 20% between any two individual allotypes which results in considerably different preferential sequence binding motifs (for an examination of this, see Hurley & Steiner, 1995). However, during the normal course of the maturation of class II MHC molecules, the digestion of Ii within the endocytic compartments leaves all $\alpha\beta$ dimers associated transiently with a nested set of peptides derived from the same single region of the Ii protein, residues 81–107, the so-called CLIP (class II MHC-associated invariant chain peptides; Rudensky *et al.*, 1991; Chicz *et al.*, 1992, 1993; Hunt *et al.*, 1992; Riberdy *et al.*, 1992; Sette *et al.*, 1992a). It was the aim of this research to address how the CLIP sequence is able to associate with so many different class II MHC allotypes, seemingly regardless of their individual ligand binding preferences.

At the commencement of this investigation, very little information on the structural details of the CLIP–class II MHC interaction was in existence. Most critically, the site on the MHC heterodimer at which CLIP interacts had not been identified unequivocally, for example, by X-ray crystallographic analysis. This was of some importance since a scenario in which CLIP associates external to the polymorphic class II MHC binding cleft, where the sequences of the α- and β-chains are more highly conserved (Kaufman & Strominger, 1982; Benoist *et al.*, 1983a; Choi *et al.*, 1983; Kaufman *et al.*, 1984; Schenning *et al.*, 1984), would account readily for the degenerate nature of CLIP binding to these molecules. However, such a model was incompatible with certain empirical data available at this time. For example, the initial description of CLIP had been made following its identification amongst antigen-derived ligands that had been eluted from the peptide-binding groove of different class II MHC allotypes (Rudensky *et al.*, 1991; Chicz *et al.*, 1992, 1993; Hunt *et al.*, 1992; Riberdy *et al.*, 1992; Sette *et al.*, 1992a). Moreover, competition binding assays had shown that CLIP and antigenic peptides were mutually exclusive in their associations with the $\alpha\beta$ dimers (Chicz *et al.*, 1992; Hunt *et al.*, 1992; Riberdy *et al.*, 1992; Sette *et al.*, 1992a). This provided compelling evidence for one common binding site upon the class II MHC molecule for these two ligands. In further support of this idea, it was observed that, despite the overall promiscuity with which CLIP associates with these molecules, the affinity of the interaction was not uniform between different MHC allotypes (Avva & Cresswell, 1994; Bangia & Watts,

1995; Liang *et al.*, 1995; Sette *et al.*, 1995). This feature had some resemblance to the binding of antigen-derived peptides to different class II MHC variants and implicated once more the peptide-binding groove as the site of CLIP interaction, since this is the primary location of allelic variation within the MHC heterodimer.

Nevertheless, not all features of the CLIP–class II MHC interaction could be reconciled with a binding site in common with antigenic peptides. For example, in addition to their unusually promiscuous binding behaviour, CLIP ligands complexed with class II MHC molecules were peculiar in their incapacity to confer resistance to subunit dissociation of the αβ dimer in the presence of the detergent, SDS, a feature highly characteristic of conventional peptide ligands (*e.g.* Germain & Hendrix, 1991; Sadegh-Nasseri & Germain, 1991; Riberdy *et al.*, 1992; Sette *et al.*, 1992a). Moreover, with regards to the allotype-dependent affinities of the CLIP–class II MHC interaction, superantigens were also reported to bind to some class II MHC variants better than others (Herrmann *et al.*, 1989; Herman *et al.*, 1990; Scholl *et al.*, 1990a, 1990b), despite it being known that they interacted primarily at conserved sites outside of the polymorphic peptide-binding cleft (Jardetzky *et al.*, 1994; Kim *et al.*, 1994). These features allowed the possibility that CLIP might bind to class II MHC molecules in a mode different from that of antigen-derived peptides. In this respect, it could be envisaged that the ability of CLIP to compete with such ligands might arise *via* an allosteric mechanism, whereby CLIP bound to a less polymorphic site on the αβ dimer some distance from the conventional peptide-binding groove and from here influenced the binding of antigenic peptides, for example, by a shift in the molecular conformation. Without further knowledge of the binding interaction at this time, this possibility could not be excluded.

A definitive means by which to elucidate the molecular basis of the promiscuity with which CLIP is able to interact with class II MHC molecules would be to dissect the specific intermolecular forces involved in binding. With this aim, Gautam and coworkers began a series of experiments in 1994 to explore the interaction of CLIP with several variants of the I-A isotype of mouse class II MHC molecules. In particular, these researchers set out to determine which specific CLIP residues were crucial for association. Using single L-alanine-substituted CLIP analogues as competitors in antigen presentation assays, Gautam and colleagues showed that the binding of CLIP to the molecules, I-A^a, I-A^k and I-A^d, was generally tolerant of such side chain substitutions. Indeed, the majority of the L-alanine-substituted CLIP bound essentially like the wild-type CLIP86–104 peptide, with relatively few side chains within this sequence responsible for modulating the binding affinity. Now published (Gautam *et al.*, 1995),

these preliminary findings provided the impetus for the subsequent investigations presented herein.

The initial task of this study was to build upon the results of Gautam *et al.* (1995) and use alternative methods of analysis to provide intersecting evidence on the role of particular CLIP residues in the interaction with mouse I-A class II MHC molecules. In this chapter, a model of degenerate CLIP binding is proposed and examined, and the virtues and limitations of each experimental approach are discussed.

3.2. Materials and Methods

The experimental procedures performed in this chapter are described fully in *Chapter 2*. Briefly, the mouse B cells lines, A20 (H-2^d), CH27 (H-2^k), M12.D (I-A^d) and 1-5.4 (I-A^u), detailed previously in *Chapter 2*, Table 2.1, were used as APC for cell-surface peptide binding assays. Mouse I-A^d molecules were purified from detergent-solubilised membrane preparations from the BALB/c-derived B cell lymphoma, A20, by immunoaffinity chromatography on an MK-D6 mAb column (§2.6). I thank Mrs Y. M. Gautam (Laboratory technician) for purifying these proteins.

L-alanine-substituted CLIP86–104 analogues are listed in Table 3.1. Figure legends state peptide concentrations used in the binding assays. Competitor peptides were tested over the concentration range 16–250 μM for cell-surface binding assays (*Chapter 2*, §2.7.2) and 25–200 μM for immunoassays with purified I-A^d (§2.7.3). Experiments to determine the relative binding affinity of peptides to purified I-A^d as a function of pH (Figures 3.4 & 3.5) were kindly performed by Mrs Y. M. Gautam.

3.3. Results

3.3.1 The binding of L-alanine-substituted CLIP analogues to cell-surface I-A class II MHC molecules

To examine the role of particular amino acid residues within the CLIP sequence for binding to I-A class II MHC molecules, Gautam *et al.* (1995) used L-alanine-substituted analogues of the CLIP86–104 peptide (Table 3.1) as competitors in antigen presentation assays with I-A-restricted T cell hybridomas. The capacity of each CLIP analogue to disrupt the productive association between peptide determinant and MHC heterodimer,

Table 3.1 *L-alanine-substituted peptide analogues of CLIP86–104.*

K86A	L-Ala	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P87A	-	L-Ala	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
V88A	-	-	L-Ala	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S89A	-	-	-	L-Ala	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Q90A	-	-	-	-	L-Ala	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M91A	-	-	-	-	-	L-Ala	-	-	-	-	-	-	-	-	-	-	-	-	-
R92A	-	-	-	-	-	-	L-Ala	-	-	-	-	-	-	-	-	-	-	-	-
M93A	-	-	-	-	-	-	-	L-Ala	-	-	-	-	-	-	-	-	-	-	-
wt CLIP86–104	K	P	V	S	Q	M	R	M	L-Ala	T	P	L	L	M	R	P	M	S	M
T95A	-	-	-	-	-	-	-	-	-	L-Ala	-	-	-	-	-	-	-	-	-
P96A	-	-	-	-	-	-	-	-	-	-	L-Ala	-	-	-	-	-	-	-	-
L97A	-	-	-	-	-	-	-	-	-	-	-	L-Ala	-	-	-	-	-	-	-
L98A	-	-	-	-	-	-	-	-	-	-	-	-	L-Ala	-	-	-	-	-	-
M99A	-	-	-	-	-	-	-	-	-	-	-	-	-	L-Ala	-	-	-	-	-
R100A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	L-Ala	-	-	-	-
P101A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	L-Ala	-	-	-
M102A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	L-Ala	-	-
S103A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	L-Ala	-
M104A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	L-Ala

Amino acid sequences of wild-type CLIP86–104 and its single L-alanine-substituted analogues are shown. Native amino acid residues are represented with a dash.

and thereby inhibit the MHC-restricted T cell response, may be interpreted as reflecting the ability of the individual monosubstituted peptides to bind to the given MHC allotype.

An alternative approach by which the efficiency of the binding interaction between a peptide and a class II MHC molecule may be assessed involves measuring the number of complexes formed between them on the surface of antigen presenting cells. These complexes may be detected readily by, first, incubating the cells with a biotin-conjugated form of the peptide, then staining with avidin which has been derivatised with the fluorochrome, fluorescein isothiocyanate (FITC). Measuring the resultant fluorescence by flow cytometry yields a signal which bears a direct relationship to the concentration of the biotinylated peptide–MHC complexes on the cell surface (Busch *et al.*, 1990).

Similar to the antigen presentation assay, the cell-surface peptide binding assay permits a wide array of peptide analogues to be tested for binding by using them as competitors, in this case against the biotinylated wild-type ligand. The ability of the 19-mer, CLIP86–104, and its two extended variants, CLIP81–104 (24-mer) and CLIP81–109 (29-mer), to compete against biotinylated CLIP86–104 for binding to the cell-surface class II MHC molecules expressed by the mouse B cell lymphomas, A20 and CH27, is shown in Figure 3.1. It is of interest to note that, for both cell lines, all three length variants showed the same degree of competition against the biotinylated peptide, *i.e.* the additional residues present on the longer CLIP ligands did not appear to contribute any further net positive binding energy to the interaction.

Using the cell-surface peptide binding assay, the contribution of individual side chains within the CLIP sequence to the binding affinity for I-A molecules was examined further, this time employing the L-alanine-substituted CLIP analogues as competitors against the biotinylated wild-type peptide, CLIP86–104. Shown in Figure 3.2 are the results of one such assay, representative of the experimental findings. Mean IC_{50} values, calculated from two independent experiments, *i.e.* the concentration of competitor peptide required to inhibit 50% of the biotinylated CLIP86–104 signal, are given atop each bar to provide a quantitative representation of the overall trends observed. Using the same I-A-expressing mouse APC lines as the original antigen presentation experiments of Gautam *et al.* (1995), the results obtained with this flow cytometry-based method (Figure 3.2) correlate well with the earlier experiments. The majority of the L-alanine-substituted CLIP analogues (solid fill pattern) competed essentially the same as the unlabelled wild-type CLIP sequence, CLIP86–104 (cross-hatched fill pattern), for binding to the cell-surface class II MHC molecules expressed by the three cell lines, 1-5.4 ($I\text{-}A^s$), CH27 ($H\text{-}2^k$) and A20 ($H\text{-}2^d$). The substitutions of the wild-type CLIP residues that deviated notably from

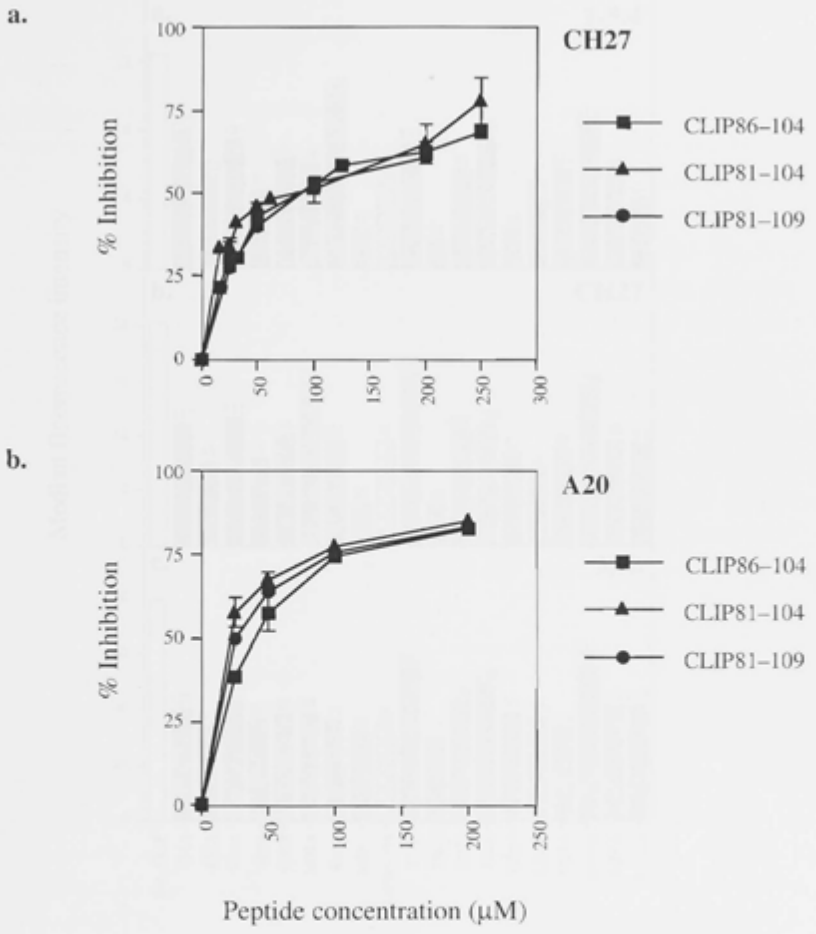


Figure 3.1 *Competition by CLIP ligands with biotinylated CLIP86–104 for binding to cell-surface class II MHC molecules at pH 7.0.*

The 19-mer wild-type peptide, CLIP86–104, and its two extended variants, CLIP81–104 (24 residues) and CLIP81–109 (29 residues), were examined for their ability to inhibit the cell-surface binding of biotinylated CLIP86–104 (50 μM) to **(a)** CH27 and **(b)** A20 cells. Data presented represent the mean percentage inhibition ± SD from two independent experiments. Cells were incubated with biotinylated peptide and competitor for 18 hours at 37°C before flow cytomteric analysis, as described in*Chapter 2*, §2.7.2.

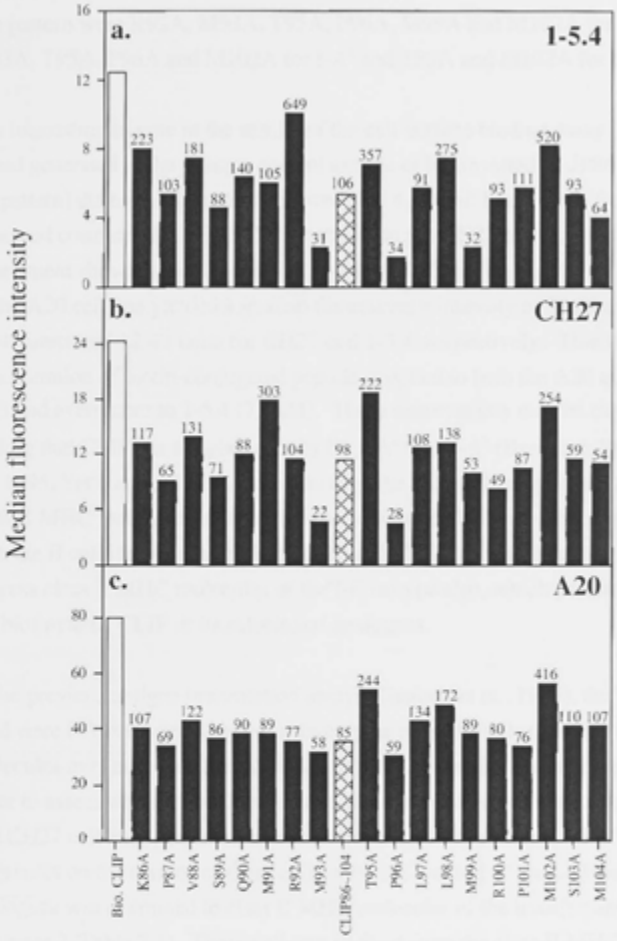


Figure 3.2 *Competition by L-alanine-substituted CLIP86–104 for cell-surface binding to I-A-expressing cell lines.*

Multiple doses of CLIP86–104 analogues with single L-alanine substitutions were tested for competition against biotinylated CLIP86–104 (1-5.4, 75 μM; CH27 & A20, 50 μM) for binding to the I-A MHC molecules of the cell lines **(a)** 1-5.4 (I-A^u), **(b)** CH27 (H-2^k) and **(c)** A20 (H-2^d). A single competitor dose which yielded ~50% inhibition of the biotinylated CLIP signal by wild-type CLIP86–104 (cross-hatched fill pattern) is shown for brevity (125 μM). All assays were perfomed twice with equivalent results, as described in *Chapter 2*, §2.7.2. Solid bars represent competition by the substituted CLIP analogues and empty bars represent the maximal biotinylated CLIP signal in the absence of competitor. Binding levels are expressed as median fluorescence intensity, from which background counts measured in the absence of biotinylated peptide have been subtracted (1-5.4, 2.94; CH27, 3.40; A20, 3.69). IC_{50} values for are given for competitors above each bar.

this pattern were R92A, M93A, T95A, P96A, M99A and M102A for I-A^a, M91A, M93A, T95A, P96A and M102A for I-A^k and T95A and M102A for I-A^d.

It is interesting to note in the results of the cell-surface binding assay (Figure 3.2) that the signal generated in the positive control sample of biotinylated CLIP86–104 alone (empty fill pattern) differed substantially between the three cell lines. Specifically, the A20 cells appeared consistently to bind CLIP better than the CH27 or 1-5.4 lines. In the experiment shown, for example, biotinylated CLIP86–104 binding to the I-A^d molecules of the A20 cell line yielded a median fluorescence intensity of 79.85 units, compared with 24.49 units and 12.46 units for CH27 and 1-5.4, respectively. This was despite the same concentration of biotin-conjugated peptide supplied to both the A20 and CH27 cells (50 μM) and even more to 1-5.4 (75 μM). These observations may be explained by the finding that CLIP has a higher affinity for I-A^d than I-A^k (Bangia & Watts, 1995; Liang *et al.*, 1995; Sette *et al.*, 1995). Alternatively, the A20 cells may express greater numbers of class II MHC molecules at their plasma membrane. In this respect, it should be noted that both the B cell lymphoma cell lines, A20 and CH27, used in this study constitutively express class II MHC molecules of the I-E isotype also, which possibly may have bound the biotinylated CLIP or its substituted analogues.

In the previous antigen presentation assays (Gautam *et al.*, 1995), the T cell hybridomas used were I-A-restricted, thereby avoiding the possibility that the presence of I-E molecules may influence the examination of CLIP binding during these experiments. In order to assess whether the fluorescence signal measured in the experiments with the A20 and CH27 cell lines presented herein reflected the association of CLIP with the I-A molecules on the surface of these cells only, the binding of the L-alanine-substituted CLIP analogues was examined to class II MHC molecules of the transfectant B cell line, M12.D (*Chapter 2*, Table 2.1). These cells are derived from the class II MHC-negative cell line, M12.C3 (Glimcher *et al.*, 1985), which has been transfected with $A\alpha^d/A\beta^d$ cDNA to express only I-A^d molecules at the plasma membrane. In a cell-surface peptide binding assay (Figure 3.3), biotinylated CLIP bound equally well to the I-A^d molecules of the M12.D cell line (a median fluorescence intensity of 33.11 units) as to the A20 cells shown previously in Figure 3.2, taking into account the different levels of expression of the cell-surface class II MHC molecules between these two cell lines on the day of the experiment (for the data shown, a median fluorescence intensity reading of 120 units for M12.D and 209 units for A20, as determined by staining with the fluoresceinated, anti-I-A^d mAb, MK-D6-FITC). Moreover, the L-alanine-substituted CLIP bound to the I-A^d molecules on the surface of the M12.D cells with an identical pattern of results to those obtained with A20. Again, mean IC_{50} values of two independent experiments are shown atop each

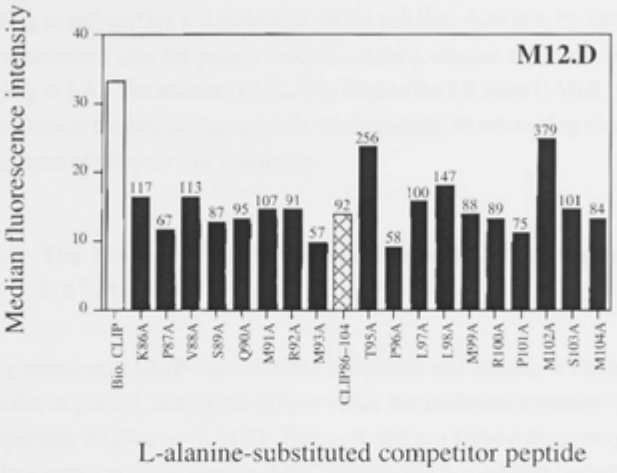


Figure 3.3 *Competition by L-alanine-substituted CLIP86–104 for cell-surface binding to M12.D cells at pH 7.0.*

Multiple doses of CLIP86–104 analogues with single L-alanine substitutions were tested for competition against biotinylated CLIP86–104 (50 μM) for binding to I-A^d class II MHC molecules on the surface of M12.D cells. Shown is a single competitor concentration (125 μM) which yielded ~50% inhibition of biotinylated CLIP binding by wild-type CLIP86–104 (cross-hatched fill pattern). The assay was performed twice with equivalent results. Cells were incubated with biotinylated peptide and competitor for 18 hours at 37°C before flow cytometric analysis, as described in *Chapter 2*, §2.7.2. Solid bars represent competition by the substituted CLIP analogues and the maximum biotinylated CLIP signal with no competitor present is depicted by the empty bar. Binding levels are expressed as median fluorescence intensity (MFI). The background signal from non-specific binding was measured in the absence of biotinylated peptide and has been subtracted from data shown (M12.D, 2.77 MFI units). IC_{50} values for are given for competitors above each bar.

bar in Figure 3.3. From these findings it may be assumed that any contribution of CLIP binding to cell-surface I-E molecules on the cell line, A20 and, by extension, CH27, does not obscure nor alter the pattern observed of the L-alanine-substituted CLIP analogues binding to I-A. The inability of CLIP to bind to the I-E class II MHC molecules under the conditions of the cell-surface peptide binding assay, an interesting observation in itself, is the subject of *Chapter 5* of this thesis.

3.3.2 The binding of L-alanine-substituted CLIP analogues to purified I-A^d at pH 5.0

The generation of CLIP–class II MHC complexes and loading of these molecules with peptides in general, both occur *in vivo* within the acidic environment of the endocytic compartments (*Chapter 1*, §1.5). However, antigen presentation assays and cell-surface binding assays examine peptide–MHC interactions in a cellular context, which excludes the possibility of probing this interaction in anything other than the neutral pH conditions needed to sustain cell survival unless the cells are fixed, for example, using paraformaldehyde (Jensen, 1990). In this study, an alternative approach was chosen by which to investigate peptide binding to class II MHC molecules at a physiologically-relevant acidic pH, namely, an ELISA-type assay with affinity-purified class II MHC proteins. This method increases the probability that the binding interactions observed involve only the $\alpha\beta$ dimers of interest, in this case, ensuring no influence upon the results of CLIP binding to I-E molecules.

As with the cell-surface binding assay, the immunoassay with purified MHC uses a biotinylated ligand to generate a signal, against which different peptide analogues may be tested as competitors. Figure 3.4 shows the ability of biotinylated CLIP86–104 to bind to mouse I-A^d, purified by affinity chromatography from the BALB/c-derived B cell lymphoma, A20. The binding of this peptide at pH 5.0 was enhanced with respect to binding at neutral pH. A similar but even more striking pH-dependent effect was seen with I-A^d binding its biotinylated antigen-derived ligand, Ova322–339 (Figure 3.5a). It is likely that a more acidic environment also serves to increase the specificity of peptide binding. This is illustrated in Figure 3.5b, where the I-E^k-restricted peptide, mCytc88–103, from the moth cytochrome *c* protein (Schild *et al.*, 1995) did not bind to the I-A^d molecules at pH 5.0 but did show some association at pH 7.0.

Using the immunoassay, the ability of the L-alanine-substituted CLIP analogues to compete with biotinylated CLIP86–104 for binding to mouse I-A^d was examined at pH

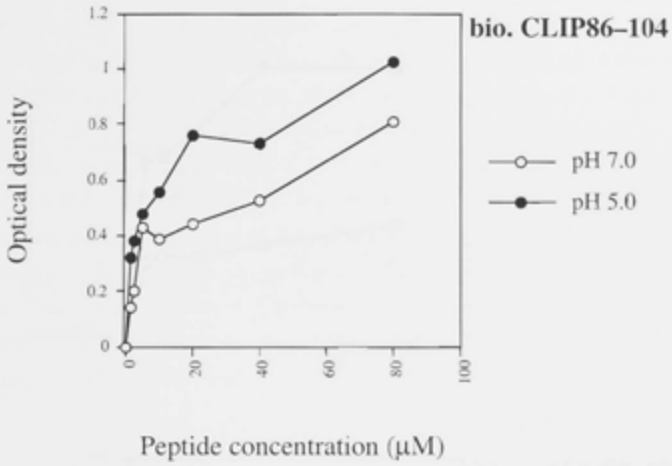


Figure 3.4 *The effect of pH on the binding of CLIP86–104 to purified I-A^d class II MHC molecules.*

Biotinylated murine CLIP86–104 was incubated with affinity-purified I-A^d proteins at 37°C for 48 hours at pH 7.0 (open circles) or pH 5.0 (closed circles), as described in *Chapter 2*, §2.7.3. I-A^d molcules were purified from the BALB/c-derived B cell lymphoma, A20, by affinity chromatography (§2.6). Results shown are representative of two independent assays. Binding levels are expressed in optical density (OD) units, read at 405 nm with a reference wavelength of 495 nm. The signal from non-specific associations was determined from the binding of biotinylated peptide in the absence of MHC molecules and has been subtracted from data shown (pH 7.0, 0.061 OD units; pH 5.0, 0.059 OD units).

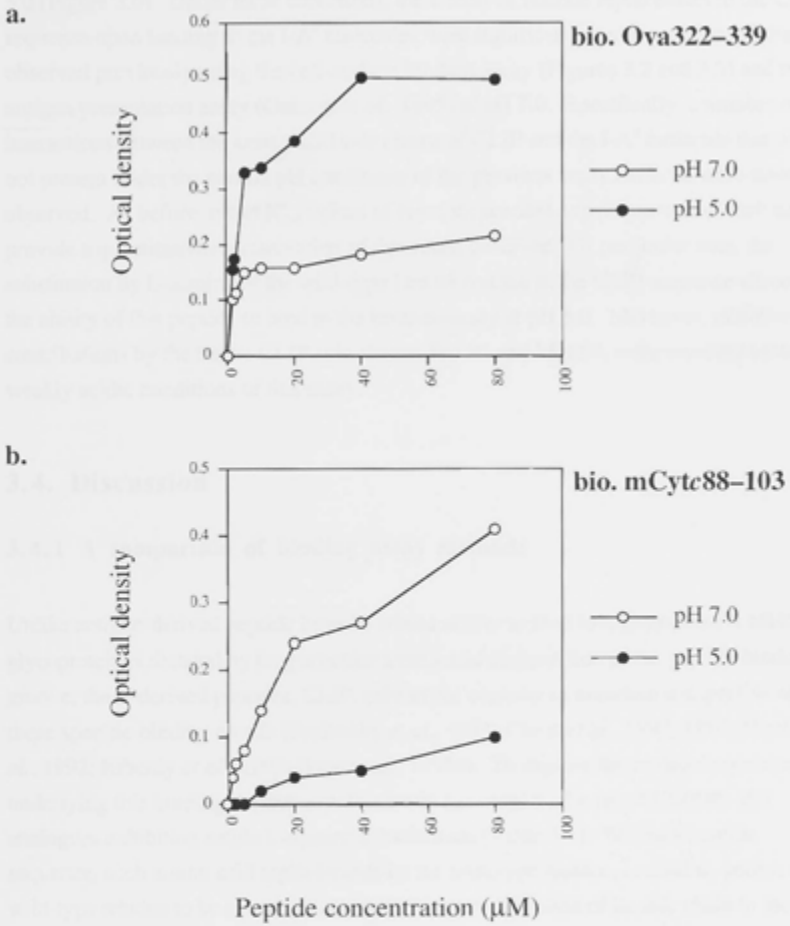


Figure 3.5 *The binding of antigen-derived peptides to purified I-A^d.*

Biotinylated peptides (**a**) Ova322–339 and (**b**) *m*Cytc88–103 were incubated with I-A^d at 37°C for 48 hours at pH 7.0 (open circles) or pH 5.0 (closed circles), as described in *Chapter 2*, §2.7.3. I-A^d molecules were purified from the BALB/c-derived B cell lymphoma, A20, by affinity chromatography (§2.6). For each peptide is shown one of two independent experiments with equivalent results. Binding levels are expressed in optical density (OD) units, read at 405 nm with a reference wavelength of 495 nm. The signal from non-specific associations was determined from the binding of biotinylated peptide in the absence of MHC and has been subtracted from data shown (pH 7.0, 0.061 OD units; pH 5.0, 0.059 OD units).

5.0 (Figure 3.6). Under these conditions, the effects of residue replacement in the CLIP sequence upon binding to the I-A^d molecules were significantly more pronounced than observed previously using the cell-surface binding assay (Figures 3.2 and 3.3) and the antigen presentation assay (Gautam *et al.*, 1995) at pH 7.0. Specifically, a number of interactions between the amino acid side chains of CLIP and the I-A^d molecule that were not present under the neutral pH conditions of the previous assay methods were now observed. As before, mean IC_{50} values of two independent experiments atop each bar provide a quantitative representation of the trends observed. Of particular note, the substitution by L-alanine of the wild-type Leu 98 residue in the CLIP sequence eliminated the ability of this peptide to bind in the immunoassay at pH 5.0. Moreover, inhibitory contributions by the native CLIP side chains, Pro 96 and Met 99, were revealed under the weakly acidic conditions of this assay.

3.4. Discussion

3.4.1 A comparison of binding assay methods

Unlike antigen-derived peptide ligands, whose ability to bind to a given class II MHC glycoprotein is dictated by the particular amino acid composition of the peptide-binding groove, the Ii-derived peptides, CLIP, exhibit the capacity to associate irrespective of these specific binding motifs (Rudensky *et al.*, 1991; Chicz *et al.*, 1992, 1993; Hunt *et al.*, 1992; Riberdy *et al.*, 1992; Sette *et al.*, 1992a). To explore the molecular mechanism underlying this binding degeneracy, this study has employed a set of CLIP86–104 analogues exhibiting single L-alanine substitutions (Table 3.1). Within a peptide sequence, such amino acid replacements by the prototype residue, L-alanine, permit each wild-type residue to be examined in turn as to the contribution of its side chain to the binding interaction. These analogues have been used to dissect the promiscuous interaction of CLIP with variants of mouse class II MHC molecules of the I-A isotype.

The binding of CLIP to the molecules, I-A^a, I-A^k and I-A^d, has been studied previously using the L-alanine-monosubstituted analogues as competitors in antigen presentation assays with I-A-restricted T cell hybridomas (Gautam *et al.*, 1995). The investigation initiated in that study has been extended here, with an examination of the binding of these side-chain substituted analogues to the same three I-A allotypes by two further experimental approaches, firstly, the analysis of the binding of these peptides to cell-surface I-A molecules and, secondly, the assessment of their interaction with affinity-purified I-A^d.

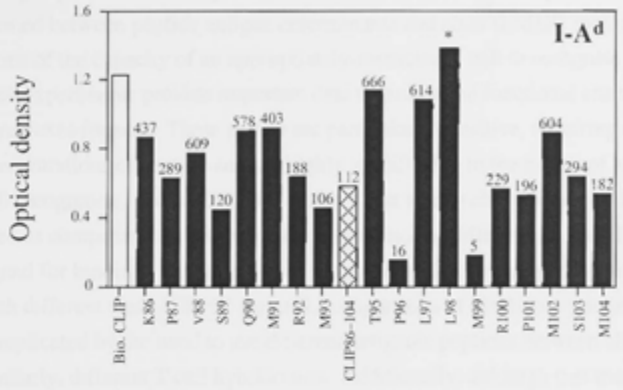


Figure 3.6 *Competition by L-alanine-substituted CLIP86–104 for binding to purified I-A^d at pH 5.0.*

Multiple doses of CLIP86–104 analogues with single L-alanine substitutions were tested for competition against biotinylated CLIP86–104 (30 μM) for binding to I-A^d molecules affinity-purified from the BALB/c- derived B cell lymphoma, A20 (*Chapter 2*, §2.6). A single competitor dose which yielded ~50% inhibition of the biotinylated CLIP signal by wild-type CLIP86–104 (cross-hatched fill pattern) is shown (200 μM). Binding levels are expressed in optical density (OD) units, read at 405 nm with a reference wavelength of 495 nm. Solid bars represent competition by the substituted CLIP analogues. The maximum biotinylated CLIP signal with no competitor present is depicted by the empty bar. Assays were performed twice with consistent results. Binding levels are expressed in optical density (OD) units, read at 405 nm with a reference wavelength of 495 nm. Non-specific signal was determined from the binding of biotinylated peptide in the absence of MHC molecules and has been subtracted from the data shown (I-A^d, 0.085 OD units). IC_{50} values for are given for competitors above each bar. An asterisk represents an IC_{50} value >1000 μM.

Antigen presentation assays determine the relative number of productive associations formed between peptide antigen determinants and class II MHC molecules, measured in terms of the capacity of an appropriately-restricted T cell to recognise them. In this way, such experiments provide important data regarding the functional character of the complexes formed. These assays are particularly sensitive, requiring only very low concentrations of peptide and are highly specific due to the nature of MHC-restricted T cell recognition. Increasing their utility, these assays also permit peptide analogues to be used as competitors to determine the importance of different structural features of the ligand for binding. However, when competition studies are performed in this manner with different class II MHC variants, comparisons of inhibitory potential may be complicated by the need to use different antigenic peptides between allotypes and, similarly, different T cell hybridomas. Additionally, although this method provides a useful way to determine the binding propensity of a particular peptide for class II MHC molecules in a whole cell model, the precise interactions between just peptide and binding groove may be obscured by downstream effects involving the T cell receptor. For example, the substitution of a glutamic acid residue for valine at position β29 in the floor of the peptide-binding site of $I\text{-}E^k$ has been shown to decrease the functional presentation of the HIV-1 gp160 determinant, T1(428–443) to a specific T cell hybridoma, however the binding of the peptide itself to the class II MHC heterodimer is unaffected (Boehncke *et al.*, 1993). By contrast, an amino acid substitution within this particular peptide sequence led to enhanced T cell stimulation as a result of an increase in the extent of binding between the peptide–MHC pair.

The flow cytometry-based cell-surface binding assay involves incubating cells with biotinylated, synthetic peptide, then staining with avidin conjugated to the fluorochrome, FITC. The fluorescence signal generated is related directly to the relative ability of the biotinylated peptide to bind to the class II MHC molecules at the plasma membrane of either mouse (Mozes *et al.*, 1989) or human cells (Rothbard *et al.*, 1989). As with the antigen presentation assay, this method allows the structural requirements of peptides for binding to be defined easily by using a series of substituted analogues as competitors, in this case, against the biotinylated signal peptide. However, because the assay operates regardless of peptide antigenicity or TCR recognition, this method has the advantage of permitting a single peptide sequence (such as the promiscuous CLIP) to be used to probe the binding interactions of different class II MHC variants. The flow cytometry assay is a comparatively rapid, reliable and simple means for screening large numbers of peptides and, regardless of day-to-day fluctuations in class II MHC expression amongst cells in culture, the method displays an extraordinary degree of reproducibility. Also, a high degree of specificity is exhibited regarding the binding of the fluoresceinated avidin to

biotinylated peptide–class II MHC complexes and with minimal background fluorescence due to the high-affinity nature of the avidin-biotin interaction ($K_d \approx 10^{-15}$ M; Green, 1975). Unfortunately, however, this assay method tends to consume large amounts of peptide and may be less sensitive than the T cell assay in detecting the influence of some amino acid substitutions on the peptide–MHC interaction.

Both the antigen presentation and flow cytometry binding assay protocols involve providing an exogenous supply of a synthetic peptide of interest to whole cells, in order to gain a measure of the ability of the peptide to associate with a certain class II MHC molecule. Monji & Pious (1997) have reported that such exogenously-provided peptides are presented as efficiently by aldehyde-fixed B lymphoblastoid APC as by unfixed cells. Furthermore, these investigators did not find that exogenous peptides bound detectably to nascent intracellular class II MHC molecules nor were the peptides provided in such a manner found to accumulate intracellularly to any great extent. Together, these results indicate that intracellular processes do not contribute extensively to presentation of exogenously-suppled peptides. Rather, the binding of such peptides appears to occur primarily, or even exclusively, to a subset of pre-existing class II MHC molecules at the plasma membrane. Given the sensibly irreversible nature of cognate peptide–class II MHC binding (*e.g.* Buus *et al.*, 1986; Lanzavecchia *et al.*, 1992; Bot *et al.*, 1996), suitable candidate αβ dimers would be only those associated weakly with peptide or heterodimers already devoid of ligand as a result of prior dissociation. Depending on cell type examined, such forms of class II MHC molecule have been estimated to represent between 1–15% of the 10^5–10^6 total cell surface molecules of any given allotype (Trucco *et al.*, 1980; Watts & McConnell, 1986; Buus *et al.*, 1988; Ceppellini *et al.*, 1989; Roche & Cresswell, 1990b). Accordingly, Busch *et al.* (1990) have calculated from a flow cytometry binding assay using the B lymphoblastoid cell line, MAJA, that the biotinylated T cell determinant, HA307–319, binds to approximately only 1% of the total number of HLA-DR1 αβ dimers estimated to be present on the surface of these cells. Although seemingly low, this number of molecules occupied with biotinylated peptide is still clearly within the limits of detection for this method. Likewise, in the context of the antigen presentation assay, the approximate 10^3–10^4 class II MHC molecules *per* cell that this percentage represents exceeds the 10–100 specific peptide-bound complexes estimated to be necessary for T helper cell stimulation and proliferation (Watts & McConnell, 1986; Demotz *et al.*, 1990; Harding & Unanue, 1990; Srinivasan *et al.*, 1991).

In contrast to the binding of exogenously-provided peptides, the bulk of processed antigen fragments *in situ* bind to class II MHC molecules intracellularly, specifically within the acidic compartments of the endocytic route (Rudensky *et al.*, 1994; Castellino

& Germain, 1995; Morkowski *et al.*, 1997). Under such conditions of high proton concentration, peptide binding has been shown to exhibit accelerated rates of association (Jensen, 1990, 1991; Harding *et al.*, 1991; Wettstein *et al.*, 1991; Mouritsen *et al.*, 1992; Sette *et al.*, 1992b). It is therefore useful to have an experimental means by which to examine the interaction of peptide with αβ dimers under conditions that mimic the physiological situation. In this study, this has been achieved using affinity-purified class II MHC glycoproteins in an enzyme-linked immunoassay. This very direct means of examining peptide–MHC binding involves capturing the class II MHC complexes with appropriate solid-phase monoclonal antibodies following an incubation period with biotinylated peptide. Bound biotin-conjugated ligand is quantified by incubation with streptavidin-HRP followed by a chromogenic substrate, such as ABTS (Jensen, 1991). This method provides an advantage over the flow cytometry and antigen presentation assays in that the binding reactions may be performed under conditions of controlled acidity, including at the low pH conditions found at the site of peptide loading *in vivo* within the endocytic compartments. Furthermore, a considerable degree of selectivity is ensured at the outset by the use of immunopurified class II MHC molecules. This increases significantly the probability that the ligand interactions observed involve only the αβ dimers of interest. The binding assay itself displays a high degree of specificity by exploiting the extremely stable, high affinity avidin–biotin interaction for detecting the peptide-bound complexes, similar to the cell-surface binding assay. This method also exhibits a high degree of sensitivity due to its avoidance of other interfering cellular processes, such as proteolysis. Moreover, only small quantities of reagents are consumed, the assay may be performed with multiple samples simultaneously and the system is compatible with the use of dimethyl sulphoxide (DMSO) to dissolve peptides that are insoluble in aqueous solutions. Critical to the success of this technique is the selection of an appropriate anti-class II MHC monoclonal antibody to capture the peptide-bound complexes — a conformation-specific antibody should be avoided as it may bias which molecules are captured. However, the experimental protocols for this assay are more involved than those of the antigen presentation or the flow cytometry cell-surface binding assay due to the requisite MHC purification step. Moreover, some binding interactions may be disrupted in the presence of particular detergents, including those used frequently to solubilise the class II MHC proteins during purification, *e.g.* Nonidet P-40 (NP-40; Fraser, 1989; Avva & Cresswell, 1994; Kropshofer *et al.*, 1995b). Nevertheless, this assay method provides a useful means for a further examination of particularly interesting peptide–MHC binding interactions, for example, those highlighted previously by a preliminary screening with the flow cytometry-based assay method. Unfortunately, time did not permit the purification of sufficient quantities of mouse I-A^k

and I-A^u proteins during this study in order to examine their interaction with CLIP by this ELISA-type method.

No one experimental method will provide a complete account of the details of an intricate and dynamic biochemical association, such as that taking place between a peptide ligand and a class II MHC glycoprotein. The three approaches described above, despite their individual limitations, each provide important information on different aspects of the interaction: the antigen presentation assays on the functional nature or immunogenicity of the complex, the cell-surface binding assays on the specific structural features required for class II MHC binding in the context of the whole cell and the immunoassay on the effects of different environmental conditions such as ionic strength, detergent and proton concentration on the peptide–class II MHC complex formation. When examined together, the individual data sets complement each other to provide a comprehensive analysis of how peptide ligands interact with different class II MHC molecules.

3.4.2 CLIP uses minimal anchor interactions for binding to I-A class II MHC molecules

The results presented herein, obtained the whole cell, flow cytometry-based assay and the immunoassay performed with purified class II MHC proteins, are largely consistent with those of the corresponding antigen presentation experiments employing I-A-restricted T cell clones performed previously by Gautam *et al.* (1995).

The results from the cell-surface peptide binding assays indicate that the majority of single side-chain substitutions within the CLIP sequence have little effect on the binding to I-A molecules (Figures 3.2 & 3.3). Indeed, in most instances, the L-alanine-substituted CLIP analogues competed for binding to the I-A αβ dimers essentially like wild-type CLIP86–104. Deviations from this trend were seen, however, with the substitution by L-alanine of the residues Arg 92, Met 93, Thr 95, Pro 96, Met 99 and Met 102 for I-A^u, Met 91, Met 93, Thr 95, Pro 96 and Met 102 for I-A^k and Thr 95 and Met 102 for I-A^d. Interestingly, not all of these particular effects were apparent to the same extent in the original antigen presentation assays of Gautam *et al.* (1995), for example, that of substituting the CLIP residue, Met 102. Similarly, some interactions highlighted in the earlier experiments of Gautam *et al.* (1995) were not as clearly defined in the cell-surface binding experiments presented herein. This is most evident in the case of the M99A analogue which was observed to bind extremely well to I-A^d and I-A^k in the antigen presentation assays. These differences likely reflect the distinct nature of the competition

established in each of these experimental methods, *i.e.* the direct competition between the L-alanine-substituted CLIP and the biotinylated wild-type form in the cell-surface binding assay, compared with that in the antigen presentation assay between the substituted analogues and a high affinity antigen-derived peptide.

Using an immunoassay technique with purified I-A^d molecules at pH 5.0, the role of certain CLIP residues in this binding interaction was even more pronounced, as illustrated by the large differences in the IC_{50} values apparent between individual side-chain substitutions (Figure 3.6). For example, the substitution by L-alanine of the residues, Pro 96 and Met 99, produced analogues which bound with very high affinity to the I-A^d molecules under these conditions. By contrast, the L98A CLIP analogue did not bind at all to these molecules at pH 5.0. In this respect, the Leu 98 residue of the CLIP sequence may be deemed an anchor residue for I-A^d at pH 5.0, *i.e.* a residue which provides positive binding energy to stabilise the resultant complex to the extent that the interaction is not sustained in its absence.

Anchor residues are a common feature of conventional class II MHC antigen-derived ligands and are largely allele-specific, for example, acting through the fulfilment of particular electrostatic requirements or hydrophobic preferences dictated by the polymorphism of the peptide-binding groove (*e.g.* Allen *et al.*, 1987; Sette *et al.*, 1987; Adorini *et al.*, 1988a; Kwok *et al.*, 1996a; Nelson *et al.*, 1996b). However, from an examination of the cell-surface binding assay data recorded at pH 7.0 (Figures 3.2 & 3.3), although certain side chains do seem to contribute some measure of positive binding energy to the interaction of CLIP with I-A^u, I-A^d and I-A^k (*e.g.* Met 102), it is apparent that no other anchor residues exist within the CLIP sequence to uphold its binding to these molecules. Similarly, in the antigen presentation experiments of Gautam *et al.* (1995), the only amino acid in the CLIP86–104 ligand that may possibly constitute an anchor residue under the conditions of this assay method is the methionine at position 91 for binding to I-A^k. Even in the immunoassays presented herein (Figure 3.6), where the higher proton concentration was found to enhance the stringency with which the modifications in the primary CLIP sequence affected the association with I-A^d, no other amino acid besides Leu 98 may be defined as an anchor. Rather, the binding of CLIP to I-A molecules using these three different experimental methodologies is notable for the number of strong inhibitory interactions, for example, through the side chains of the residues, Met 93 (I-A^u and I-A^k, Figure 3.3, Gautam *et al.*, 1995), Pro 96 (I-A^s, Figure 3.3; I-A^k, Figure 3.3, Gautam *et al.*, 1995; I-A^d, Figure 3.6) and Met 99 (I-A^u, Figure 3.3; I-A^k, Gautam *et al.*, 1995; I-A^d, Figure 3.6, Gautam *et al.*, 1995). This behaviour has been investigated for the wild-type CLIP methionine residues at positions 93 and 99

for binding to I-A molecules and determined to be a consequence of steric hindrance arising from the bulk of their long aliphatic side chains (Gautam *et al.*, 1995).

3.4.3 A model for the degenerate binding of CLIP to class II MHC molecules

The experiments reported herein identify yet another characteristic shared between CLIP and antigen-derived peptides: the ability of certain peptide side-chain substitutions to modulate the binding affinity of the interaction with class II MHC molecules. At the time, this finding added to the accumulating experimental evidence supporting the MHC peptide-binding groove as the common binding site of these two types of ligand. This was eventually confirmed in November 1995 with the publication of the crystal structure of a single CLIP ligand bound to the human class II MHC molecule, HLA-DR3 (DRA/DRB1*0301; Ghosh *et al.*, 1995). In this analysis, CLIP was identified within this site with its downward-oriented side chains positioned in the pockets of the cleft in a manner almost identical to that observed in the previous study of the antigenic influenza virus haemagglutinin peptide, HA306–318, complexed with HLA-DR1 (DRA/DRB1*0101; Stern *et al.*, 1994).

The results of the L-alanine substitution experiments presented above and previously (Gautam *et al.*, 1995) have a number of implications for the binding of CLIP within the class II MHC peptide-binding groove. For example, the absence of optimal anchor residues within this peptide sequence, with a tendency instead for certain side chains to be involved in strained interactions at the binding interface, means that this ligand would be unlikely to form highly stable complexes with the αβ dimers. This provides a likely explanation for the inability of CLIP to form complexes with I-A molecules that are able to withstand denaturation in the presence of SDS detergent (Gautam *et al.*, 1995; Stebbins *et al.*, 1996; Weenink *et al.*, 1997), namely, that insufficient binding energy is present to drive the conformational rearrangement into the highly-stable compact dimer characteristically seen when class II MHC molecules are loaded with conventional cognate antigen-derived peptides. The probability that CLIP–I-A complexes are not highly stable is also of considerable functional significance. With each newly-synthesised class II MHC molecule that enters the antigen presentation pathway being unable to bind peptide antigen until it is free of CLIP (Roche & Cresswell, 1990b, 1991), a strong interaction between this ligand and the αβ dimer would be undesirable. Consistent with this, the mutation in intact Ii of Met 93 or Met 99 to L-alanine has been shown to inhibit antigen presentation by some mouse class II MHC allotypes (Gautam *et al.*, 1997).

Most significantly, the absence of notable allele-specific anchor side chains within the CLIP sequence in the interaction with mouse I-A molecules suggests a mechanism for the degenerate binding of this peptide to the many different class II MHC variants. Through the fulfilment of such contacts, an antigen-derived peptide typically becomes restricted in the subset of MHC allotypes to which it may bind. By contrast, the residues of the CLIP sequence may occupy the pockets of any I-A binding groove, albeit suboptimally. It would appear that this ligand concertedly minimises both highly-favourable and extremely negative side-chain interactions to offer a good compromise for binding to many different αβ dimers.

In agreement with this model of the degenerate binding of mouse CLIP, Malcherek *et al.* (1995) and Geluk *et al.* (1995) have examined the binding of human CLIP to several variants of the HLA-DR class II MHC isotype and find it also to lack major allele-specific contacts. Consequently, Malcherek *et al.* (1995) proposed that the CLIP ligand achieves promiscuous interactions with HLA-DR molecules by exhibiting a 'supermotif', a sequence which is compatible with the binding motifs of many different class II MHC allotypes. Interestingly, however, this supermotif for HLA-DR does feature the use of anchor residues, in the form of the three conserved CLIP methionine residues at positions 91, 93 and 99. Specifically, these investigators determined that the substitution of Met 91 by L-alanine decreased markedly or eliminated the binding to all the molecules examined (HLA-DR1, DR2, DR3 (formerly HLA-DR17) and HLA-DR7). Likewise, the CLIP Met 93 residue was shown to provide a favourable component to the interaction with HLA-DR1 and DR3, and Met 99 appears to play a similar anchoring role to HLA-DR2, DR3 and DR7 molecules (The results of these two studies disagree over whether this residue is also provides positive binding energy to the interaction with HLA-DR1). These finding are not necessarily in conflict with the mode of degenerate binding proposed above for the association of CLIP with I-A molecules however, since these methionine anchors are clearly promiscuous, accepted by most HLA-DR molecules by representing a compromise of the particular side-chain anchors actually preferred at each of these positions (see below, §3.4.5). In this sense, these residues are still suboptimal for binding to these molecules and, consistent with this, the majority of the resultant CLIP–HLA-DR complexes remain unable to traverse the activation energy barrier of the conformational change to form the compact dimer to thereby achieve stability in the presence of SDS detergent (Riberdy *et al.*, 1992; Sette *et al.*, 1992a; Hitzel & Koch, 1996).

3.4.4 The interaction of CLIP side chains with I-A class II MHC molecules

The publication of the crystal structure of CLIP complexed with the human class II MHC molecule, HLA-DR3, provided important spatial details on the localisation of every residue of the CLIP sequence within the peptide-binding groove (Ghosh *et al.*, 1995). X-ray crystallographic data are now also available for the three-dimensional structure of I-A^k associated with the antigenic hen egg lysozyme peptide, HEL50–62 (Fremont *et al.*, 1998b), and of I-A^d molecules complexed either with a peptide derived from hen egg ovalbumin (Ova323–339) or from influenza virus haemagglutinin (HA126–138; Scott *et al.*, 1998). The comparative structure of the I-A^u heterodimer may be extrapolated from that of I-A^k since these molecules differ by only six residues in the α-chain and ten in the β-chain, all of which are located within the domains that contribute to the peptide-binding groove (Davis *et al.*, 1989). Homology modelling has also been used to examine the interaction between these different I-A class II MHC molecules and their cognate antigen-derived peptide ligands, for example, I-A^k with the hen egg lysozyme peptide, HEL52–61 (Weber *et al.*, 1998), I-A^d with the mouse IgG2a^b heavy chain allopeptide determinant, γ2a^b435–451 (Bartnes *et al.*, 1997), I-A^u with the NH_2-terminal peptide of myelin basic protein (MBP), Ac1–11 (Lee *et al.*, 1998) or simply just the I-A^u and I-A^k molecules alone (Tate *et al.*, 1995; Nelson *et al.*, 1996a, 1996b; Nydam *et al.*, 1998). This technique has been applied previously in an attempt to visualise the interaction of CLIP with these molecules as well (Lee & McConnell, 1995; Liang *et al.*, 1996). Together, this information may be used to examine features of the model of degenerate CLIP binding proposed above and to gain insight into the molecular basis of the effects observed in the I-A binding studies presented herein when certain of the amino acid side chains of this ligand were substituted with L-alanine.

Extrapolation of the structural details between different class II MHC variants is aided by the high overall degree of conservation observed in the primary amino acid sequence outside the polymorphic pockets of the peptide-binding groove (Kaufman & Strominger, 1982; Benoist *et al.*, 1983a; Choi *et al.*, 1983; Kaufman *et al.*, 1984; Schenning *et al.*, 1984). Consistent with this, the X-ray crystallographic structures of I-A^k (Fremont *et al.*, 1998b) and I-A^d (Scott *et al.*, 1998) closely resemble those of other class II MHC molecules described previously (HLA-DR3, Ghosh *et al.*, 1995; HLA-DR1, Brown *et al.*, 1993; Stern *et al.*, 1994; HLA-DR4, Dessen *et al.*, 1997; I-E^k, Fremont *et al.*, 1996). The I-A peptide-binding groove encompasses a nine-residue stretch of the bound peptide (P1–P9) and exhibits five pockets in which the side chains of the ligand are accommodated at relative positions, P1, P4, P6, P7 and P9. The intervening peptide

residues, P2, P3, P5 and P8 are oriented sideways across the groove or project outwards from this site to establish TCR contact points (*Chapter 1*, Figure 1.4).

Not seen in the previous human HLA-DR and mouse I-E^k structures though, the two I-A heterodimers show an unusual β-bulge at the base of the P4 pocket in the β-sheet platform which lines the floor of the peptide-binding groove. This feature arises from an insertion in the I-A/HLA-DQ α-chain sequence of a glycine residue in the α_1 domain at position 9a relative to the canonical structures of I-E/HLA-DR and is likely to change a number of interactions, not only between the α- and β-chains, but also with the bound peptide. Specifically, it causes the central region of this ligand (between residues P3–P6) to be drawn deeper into the groove, with the C-terminus (residues P9–P11) extending more upwards. Another I-A-conserved amino acid insertion relative to I-E/HLA-DR molecules occurs in the β1 domain at position 84a, lying at the end of the H2b helix (residues β79–β84a) which flanks the P1 binding pocket (*Chapter 1*, Figure 1.4). A deletion is also present in the molecules, I-$A^{k,f,s,u,g7}$ at residues β65 and β66. In the X-ray crystallographic structures of I-A^d (Scott *et al.*, 1998), I-E^k (Fremont *et al.*, 1996), HLA-DR1 (Brown *et al.*, 1993; Stern *et al.*, 1994), HLA-DR3 (Ghosh *et al.*, 1995) and HLA-DR4 (Dessen *et al.*, 1997), these particular residues lie at the junction between the H1 (β52–β64) and H2a helices (β65–β77), contributing to a high crest above the peptide-binding groove which represents a likely interaction site for TCR and monoclonal antibodies. In this respect, the significant reduction of this topological feature in the crystal structure of I-A^k is of some interest given the distinct patterns of mAb reactivities observed between the two groups of molecules, I-$A^{k,f,s,u,g7}$ and I-$A^{d,b,q}$ (*Chapter 2*, Table 2.4; discussed further in *Chapter 6*, §6.4.5). Throughout this thesis, I-A class II MHC residues are numbered according to the secondary structure-based scheme proposed by Fremont *et al.* (1998b) in order to be consistent with previous alignments of I-E and HLA-DR (Kabat, 1991). Further conformational features of class II MHC-bound peptides are discussed in *Chapter 4*.

Crystallographic analysis of the CLIP–HLA-DR3 complex revealed the CLIP ligand to be aligned with its Met 91 residue positioned in the first major pocket at relative position, P1 (Ghosh *et al.*, 1995). An identical binding register was predicted to be the most energetically-favourable for the binding of this ligand to I-A^d, I-A^u and I-A^k (Lee & McConnell, 1995). Using this alignment, Table 3.2 shows a summary of the interactions made by CLIP side chains with the pockets of the three I-A variants, based upon the experimental results presented herein (Figures 3.2, 3.3 & 3.6). Structural data from X-ray crystallographic analyses and homology modelling are also shown, as is information on points of polymorphism between these molecules (Davis *et al.*, 1989) and existing

Table 3.2 *The interaction of CLIP side chains with the pockets of the I-A peptide-binding groove.*

Shown are the amino acid residues whose atoms contribute to the major polymorphic pockets of the peptide-binding grooves of the class II MHC molecules, I-A^k, I-A^d and I-A^s, as determined by X-ray crystallographic analysis and/or homology modelling of these structures (Lee & McConnell, 1995; Tate *et al.*, 1995; Liang *et al.*, 1996; Nelson *et al.*, 1996; Bartnes *et al.*, 1997; Fremont *et al.*, 1998b; Lee *et al.*, 1998; Nydam *et al.*, 1998; Scott *et al.*, 1998; Weber *et al.*, 1998). Within each pocket, these residues form either hydrogen bonds or van der Waals interactions with the atoms of the amino acid side chains of a bound peptide. Throughout this thesis, class II MHC residues are numbered according to the secondary structure-based sequence alignment of the α_1 and β_1 domains of the molecules, I-A^k, I-E^k and HLA-DR, reported by Fremont *et al.* (1998b). The primary amino acid sequences of the α_1 and β_1 domains of the different I-A molecules are taken from Davis *et al.* (1989). Polymorphic residues between the I-A molecules are italicised. Those underlined provide a specific physicochemical property to the pocket to govern its overall peptide side-chain preference. For each I-A allotype, the peptide residues commonly found at each of these positions have been taken from alignments of naturally-processed ligands (Nelson *et al.*, 1996; Bartnes *et al.*, 1997; Fremont *et al.*, 1998b; Lee *et al.*, 1998; Scott *et al.*, 1998). The individual side chains of the native CLIP sequence that bind at these positions are shown in italics beneath the respective pocket number.

[a]The nature of the interaction of the native CLIP side chains is summarised from the cell-surface binding assays (pH 7.0; Figures 3.2 & 3.3) and immunoassays (pH 5.0; Figure 3.6) presented in this chapter.

[b]βAsp 57 is mutated to a serine residue in the I-A^{g7} class II MHC molecule from the non-obese diabetic (NOD) mouse. The presence of a non-Asp residue at this position has been linked to the development of autoimmune diabetes in both these animals and in humans. The effects of this substitution on the peptide-binding properties of the I-A^{g7} molecule are examined in *Chapter 6* of this thesis.

⚓ represents an anchor side chain within the CLIP sequence, such that binding to the I-A molecule is not sustained if this residue is substituted with L-alanine.

✓ represents the provision of positive binding energy by this CLIP side chain at the binding interface.

↯ represents the provision of negative binding energy by this CLIP side chain at the binding interface.

— represents no notable contribution of binding energy by the CLIP side chain to the interaction with I-A or, in the case of the wild-type CLIP residue at the P4 position, Ala 94, where this side chain was not examined by substitution analysis.

Allotype	P1 *Met 91*	P4 *Ala 94*	P6 *Pro 96*	P7 *Leu 97*	P9 *Met 99*
I-A^k	*αPhe 24, αLeu 31,* αPhe 32, αTrp 43, *αArg 52, αArg 53,* αPhe 54, βAsn 82, *βThr 85*	αTyr 9, αGly 9a, *αAsn 62, βPhe 11, βPro 13, βLeu 26, βIle 28,* βGlu 74, *βVal 78*	*αThr 11, αAsn 62, αThr 65, αGly 66, αAsn 69, βHis 9, βPhe 11,* βTyr 30	*αThr 65, αAsn 69, βIle 28,* βTyr 30, *βTyr 47, βTrp 61,* βGln 64, *βTyr 67, βArg 70*	*αHis 68, αAsn 69, αIle 72, αLeu 73,* αArg 76, *βTyr 37, βAsp 57^b, βTrp 61*
Pocket character:	medium, amphiphilic	large, hydrophobic	medium, polar	shallow, hydrophobic	medium, amphiphilic
Residues observed:	*e.g.* Asp, Glu, Thr, Cys	*e.g.* Ile, Val, Leu, Asn	*e.g.* Glu, Gln	no basic residues	*e.g.* Ser, Thr, Ala, Gly
[a]CLIP interaction:	✓	—	↗	—	—
I-A^d	*αHis 24, αLeu 31,* αPhe 32, αTrp 43, *αIle 52, αLeu 53,* αPhe 54, βAsn 82, *βPro 85*	αTyr 9, αGly 9a, *αAsn 62, βPhe 11, βGly 13, βThr 28,* βGlu 74, *βAla 78*	*αAsn 62, αAla 65,* αGlu 66, *βVal 9, βPhe 11,* βTyr 30	*αAsn 69, βThr 28,* βTyr 30, *βTrp 61, βIle 67,* βThr 71	*αHis 68, αAsn 69, αIle 72,* αArg 76, *βVal 38, βAsp 57^b,* βTyr 60, *βTrp 61*
Pocket character:	large, unrestricted	medium, hydrophobic	shallow, amphiphilic	shallow, hydrophobic	medium
Residues observed:	*e.g.* Tyr, Thr, Glu, Ser	*e.g.* Val, Ile, Leu, Ala	*e.g.* Ala, Val, Ser	*e.g.* Ala, Leu, Gly, Val	*e.g.* Ala, Ser, Val
[a]CLIP interaction:	—	—	↗	—	↗
I-A^u	*αPhe 24, αLeu 31,* αPhe 32, αTrp 43, *αArg 52, αSer 53, βTyr 81, βThr 85*	αTyr 9, *αAsn 62, βPro 13, βTyr 26, βThr 28,* βGlu 74	*αIle 10, αAsn 62, αThr 65, αGly 66, βVal 9, βPhe 11,* βTyr 30	*αAsn 69, βThr 28,* βTyr 30, *βTyr 47, βTyr 61, βTyr 67, βArg 70,* βGlu 74	*αVal 72,* αArg 76, *βLeu 38, βAsp 57^b, βTyr 61*
Pocket character:	large	shallow, solvent-exposed	deep, hydrophobic	shallow, solvent-exposed	medium
Residues observed:	*e.g.* Val	*e.g.* Ser	*e.g.* Phe, Tyr	*e.g.* Arg	*e.g.* Ser
[a]CLIP interaction:	—	—	↗	—	↗

knowledge of their individual amino acid side-chain preferences, taken from the alignment of known I-A-binding peptides and naturally-processed ligands (Nelson *et al.*, 1996b; Bartnes *et al.*, 1997; Fremont *et al.*, 1998b; Lee *et al.*, 1998; Reizis *et al.*, 1998; Scott *et al.*, 1998). In this way, the likely molecular basis for the effects observed may be assessed.

An alignment of known I-A^k-binding peptides, for example, shows an overwhelming preference at the P1 position for an aspartic acid residue (Nelson *et al.*, 1996b; Fremont *et al.*, 1998b). Indeed, the replacement of this anchor residue by an alanine in the HEL46–61 peptide has been shown to cause a 130-fold reduction in its ability to compete for binding to the I-A^k molecule and a concomitant reduction in the SDS stability of the complex from 96% to 6% (Nelson *et al.*, 1996b). This decrease in binding capacity may be recovered to some extent by restoring at least the carboxy- portion of the Asp side chain in the form of an asparagine residue. In explanation of these effects, the X-ray crystallographic analysis of the HEL-I-A^k complex has revealed that an aspartic acid side chain in the P1 pocket exhibits a near-perfect fit and forms a stabilising salt bridge with the guanidinium group of the αArg 52 residue. Meanwhile, the aliphatic portion of the Asp (or Asn) side chain makes a number of hydrophobic interactions with both the αPhe 24 and αPhe 54 residues within this site. The ability of the CLIP Met 91 residue to provide positive binding energy to the interaction with I-A^k (Figure 3.2; Gautam *et al.*, 1995) would thus appear to arise from its likely capacity to fulfil these latter interactions. By contrast, no such favourable contacts appear to be made between this side chain and the larger P1 pocket of the I-A^u molecule (Figure 3.2) nor with the I-A^d molecule unless at pH 5.0 (Figures 3.2, 3.3 & 3.6).

The role of the native CLIP residue that corresponds to the I-A P4 pocket was not examined by substitution analysis in this study, being already L-alanine (Ala 94). However, it is interesting to note that some effects of substituting the adjacent Met 93 residue were observed. The P3 binding site does not typically constitute a major pocket because it is largely just a hydrophobic surface from which the corresponding peptide side chain is oriented sideways or away from the peptide-binding groove (Fremont *et al.*, 1998b; Lee *et al.*, 1998; Scott *et al.*, 1998). It has been suggested, however, that the local plasticity of the class II MHC molecule may allow for a peptide side-chain to form an interaction at this site as an alternative to that at the P4 pocket (Rammensee *et al.*, 1995; Weber *et al.*, 1998). This is likely to be of particular significance in the event of an unsuitable anchor residue at P4. In the experiments presented herein, an inhibitory effect of the Met 93 side chain was observed upon the binding of CLIP to I-A^u and I-A^k (Figure 3.2). Such an effect was also apparent in the antigen presentation experiments of Gautam

et al. (1995) and, by examining the binding of further Met 93 substituted CLIP ligands, these authors were able to show this to be a consequence of steric conflict at the P3 site. Consistent with this, small, non-polar residues are typically found at this position in naturally-processed ligands of the I-A class II MHC molecules (*e.g.* for the HEL–I-A^k crystal structure, Gly 54; for I-A^d, Ala 326 from Ova323–339, Gly 130 from HA126–138; for I-A^u, Ala 1 from MBP *Ac*1–11; Fremont *et al.*, 1998b; Lee *et al.*, 1998; Scott *et al.*, 1998).

At the P6 pocket, all three I-A allotypes examined in this study showed that the native CLIP residue at this position, Pro 96, contributed negative binding energy to the interaction (Figures 3.2, 3.3 & 3.6). Although the exact nature and size of this pocket differs between these molecules, all appear to be unsuitable for accommodating the rigid, cyclic amino acid side chain. Subsequently, Liang *et al.* (1996) have created CLIP analogues which exhibit even greater affinity for the I-A^k and I-A^u molecules by targeting amino acid substitutions at the P6 position to their individual binding preferences, Glu and Tyr, respectively.

At the P7 pocket of the I-A^u molecule, Gautam *et al.* (1995) found previously an inhibitory interaction with the Leu 97 residue of the CLIP ligand. Although not apparent in the cell-surface binding assays presented herein (Figure 3.2), this finding has been predicted to arise from an I-A^u-specific polymorphism at position β61 (Trp → Tyr) which may create a shift inwards of the extremities of this site to favour a smaller side chain (Lee & McConnell, 1995). In most class II MHC molecules, however, the P7 pocket is noted to be shallow and frequently unrestricted in its amino acid preference. The side chain of the bound peptide at this position is typically oriented sideways towards the α-helical walls of the peptide-binding groove rather than downwards towards the β-sheet floor as in most side chain–pocket interactions (Fremont *et al.*, 1998b; Scott *et al.*, 1998). This would account for the passive accommodation of the Leu 97 CLIP side chain within the I-A^k and I-A^d molecules (Figures 3.2 & 3.3).

Lastly, the inhibitory nature of the native CLIP residue, Met 99, at the P9 pocket for binding to I-A molecules is also the result of steric strain at this position (Figures 3.2 & 3.6). Data from the X-ray crystallographic structures and homology models of these molecules would indicate that the respective pockets are simply not large enough to accommodate this bulky side chain (Lee & McConnell, 1995; Fremont *et al.*, 1998b; Lee *et al.*, 1998; Scott *et al.*, 1998). In agreement with this, Gautam *et al.* (1995) have shown that a progressive decrease in the length of the amino acid side chain at the CLIP position 99 creates a corresponding increase in the capacity of these ligands to bind. The P9

pocket of I-A^u has been predicted to be smaller than that of the I-A^k and I-A^d molecules as the result of an I-A^u-specific polymorphism at position β38 (Leu in I-A^u; Val in I-A^d and I-A^k; Lee & McConnell, 1995). X-ray crystallographic analysis of this site in the HEL–I-A^k complex has also revealed an elaborate hydrogen bonding network to be in place between the peptide Ser 60 side chain and the residues of the MHC groove, including βAsp 57 whose carboxylate group also forms a salt bridge with the αArg 76 residue at the base of this pocket (Fremont *et al.*, 1998b). The presence of the large Met 99 side chain within the P9 pocket may destabilise this network and/or weaken the important salt bridge interaction.

The remaining wild-type CLIP residues within the nine-residue peptide-binding motif, Arg 92 (P2), Thr 95 (P5) and Leu 98 (P8) are all predicted to be oriented away from the class II MHC peptide-binding groove (Ghosh *et al.*, 1995; Lee & McConnell, 1995). It is therefore unexpected that they should be able to provide some measure of positive binding energy to the interaction with the different I-A molecules. Nevertheless, the Arg 92 residue is favourable to the interaction of CLIP with I-A^u and a similar effect is noted through Leu 98 with I-A^d. The side chain of Thr 95 appears to provide positive binding energy to all three CLIP–I-A complexes (Figure 3.2, 3.3 & 3.6). Similarly, the Met 102 residue of the CLIP ligand appears to interact favourably with each of these molecules yet from crystallography is predicted to lie *outside* of the groove (Ghosh *et al.*, 1995; Lee & McConnell, 1995). In this respect, it is possible that the atoms of each of these different CLIP side chains may still make contact with the I-A molecules beyond the immediate bounds of the polymorphic pockets. For example, in the X-ray crystallographic analysis of the CLIP–HLA-DR3 complex, the aliphatic portion of the methylene group of the Arg 92 residue is noted to interact with the surface of the αβ dimer. This is particularly interesting with regards to the model proposed above to account for the promiscuous binding of CLIP to I-A class II MHC molecules (§3.4.3), since it would suggest that although the CLIP ligand lacks anchor residues to bind within the major pockets of the binding groove, favourable contacts may be formed elsewhere on the class II MHC molecules where the amino acid sequence is more highly conserved. Alternatively, the decrease in binding of the CLIP ligand observed when these residues are substituted with L-alanine may arise simply from effects being transmitted elsewhere within the peptide, for example, at adjacent pocket-binding residues. Clearly, it is not always possible to determine the molecular processes underlying an experimental observation from either an homology model or X-ray crystallographic structure.

3.4.5 The different roles of the conserved methionine residues of CLIP for binding to I-A *versus* HLA-DR class II MHC molecules

The conserved methionine residues within the CLIP sequence at positions 91, 93 and 99, are largely inhibitory in the binding interaction with I-A class II MHC molecules (Figure 3.2 & 3.6). By contrast, these residues provide anchoring contributions when associated with different HLA-DR variants (Geluk *et al.*, 1995; Malcherek *et al.*, 1995). This latter phenomenon is likely to be facilitated by the reduced extent of polymorphism in the HLA-DR peptide-binding groove as a result of these molecules sharing a common, non-polymorphic α-chain (*e.g.* Korman *et al.*, 1982; Larhammar *et al.*, 1982; Lee *et al.*, 1982; Das *et al.*, 1983; Rothbard & Gefter, 1991). At the P1 pocket in which the CLIP residue Met 91 binds, for example, almost all HLA-DR variants share a common preference for a large, hydrophobic anchor (Rammensee *et al.*, 1995; Chelvanayagam, 1997). At this site, the exact nature of side chain preferred is defined by the dimorphic β86 residue (Busch *et al.*, 1991; Demotz *et al.*, 1993; Newton-Nash & Eckels, 1993; Verreck *et al.*, 1993). Typically, if glycine is present at the β86 position, the HLA-DR heterodimer will bind an aromatic side chain in the P1 pocket preferentially, as seen with HLA-DR1 (Stern *et al.*, 1994; Rammensee *et al.*, 1995) and HLA-DR4 (DRA/DRB*0401; Dessen *et al.*, 1997). In the case of βVal 86, an aliphatic side chain is favoured, as with HLA-DR3 (Ghosh *et al.*, 1995; Rammensee *et al.*, 1995). The ability of the Met 91 CLIP to act as a promiscuous anchor thus lies in the capacity of its bulky-but-flexible, hydrophobic side chain to fulfil both of these preferences satisfactorily (Geluk *et al.*, 1995; Malcherek *et al.*, 1995). Indeed, the interactions at this site are so favourable between this ligand and HLA-DR1 that, unlike other CLIP-bound class II MHC, CLIP–HLA-DR1 complexes are readily able to resist subunit dissociation in the presence of SDS detergent (Chicz *et al.*, 1992). However, if the amino acid at position β86 or the adjacent β85 residue in the HLA-DR1 molecule is substituted, a dramatic decrease in the overall binding affinity of CLIP is observed and the stability of the αβ dimer in SDS is lost (Wu & Gorski, 1996).

In a manner similar to the Met 91 residue, the Met 93 side chain of the human CLIP ligand is also able to form favourable interactions with both HLA-DR1 and DR3 because these molecules are alike in having a shallow, non-polar P3 binding site. In fact, it is likely that the interaction of the Met 93 CLIP side chain at this site in HLA-DR1 is not altogether different from that seen with the methylene group of the Lys 310 residue in the X-ray crystallographic analysis of this molecule complexed with the HA306–318 peptide (Stern *et al.*, 1994). Likewise, HLA-DR1 and DR3 share a common preference for a hydrophobic anchor to bind in the P9 pocket (Rammensee *et al.*, 1995), thereby accounting for the promiscuous anchor interaction of the Met 99 CLIP residue at this

position (Geluk *et al.*, 1995; Malcherek *et al.*, 1995). Consistent with this argument, crystallographic analysis has indeed revealed that the CLIP anchor side chains, Met 91, Met 93 and Met 99, are each accommodated in the pockets of the HLA-DR3 molecule that exhibit the greatest proportion of their surface area formed by conserved or conservatively-substituted amino acids (Ghosh *et al.*, 1995).

An examination of CLIP binding to the mouse HLA-DR homologue, I-E, which similarly displays a non-polymorphic α-chain is presented in *Chapter 5* of this thesis. In the present chapter, it is instead more relevant to compare the CLIP–I-A interaction with the binding of this ligand to variants of the I-A human homologue, HLA-DQ. The findings with such molecules are strongly consistent with those presented herein with the I-A class II MHC. Specifically, an examination of CLIP binding to HLA-DQ2, DQ7 and DQ9 molecules has revealed that side-chain anchor contacts do not tend to be a feature of this interaction, despite being a property of the binding of antigen-derived peptides (Vartdal *et al.*, 1996; Raddrizzani *et al.*, 1997). Of further interest, the binding of CLIP to the HLA-DQ variants was found to be of low affinity and subsequent modelling studies with HLA-DQ7 have highlighted a number of significant differences within the peptide-binding grooves of HLA-DQ molecules relative to HLA-DR which are likely to be unfavourable for certain CLIP side-chain interactions. In some HLA-DQ molecules, for example, the β86 polymorphism within the P1 pocket dictates a specific preference for a polar residue (Reed *et al.*, 1997). In this instance, it may be envisaged that the Met 91 residue of the CLIP sequence would be particularly unfavourable for interacting at this site.

The differing roles of the conserved methionine residues within the CLIP sequence between the I-A and HLA-DR molecules highlights an important point regarding the interpretation of X-ray crystallographic data. Although highly informative, by their very nature such structures cannot furnish all the answers — it is not possible for all the biochemical properties of a dynamic interaction between two molecules to be understood solely from the details of a single static structure. This also holds true for hypothetical three-dimensional structures derived by computer modelling. Experimental data remain essential in order to verify theoretical conclusions. This latter point is perhaps best illustrated by considering the homology models constructed by Lee & McConnell (1995). Prior to publication of the X-ray crystallographic analysis of the CLIP–HLA-DR3 complex (Ghosh *et al.*, 1995), Lee & McConnell (1995) performed a computer simulation of the binding of CLIP within the cleft of HLA-DR3 and the three mouse allotypes, I-A^d, I-A^k and I-A^u, based on the co-ordinates of the HA peptide–HLA-DR1 crystal structure (Stern *et al.*, 1994). However, upon obtaining an alignment of the Met 91 and Met 99 CLIP side chains accommodated within the P1 and P9 pockets, respectively, these

investigators concluded that these residues were anchors for all the different class II MHC variants. Moreover, despite claiming to have assessed the accuracy of their models from the L-alanine substitution binding data of Gautam *et al.* (1995), Lee & McConnell (1995) reported that no deleterious contacts were present between this peptide and the different MHC molecules that they examined. While this is indeed an accurate assessment of the situation with HLA-DR molecules (Geluk *et al.*, 1995; Malcherek *et al.*, 1995), the data provided herein and previously (Gautam *et al.*, 1995) have shown that this is clearly not the case for CLIP binding to mouse I-A class II MHC molecules.

Nevertheless, sometimes even empirical data may be interpreted incorrectly. This time using an experimental approach, the same group of Lee and coworkers examined the effects of replacing the wild-type CLIP residues Met 91 and Met 99 with the amino acid, L-leucine (Liang *et al.*, 1996). Such substitutions were found to cause large reductions in the stability of the CLIP–I-A complexes relative to those formed with the wild-type ligand. In this respect, the data presented herein agree with the findings of Liang *et al.* (1996), that the insertion of the less-flexible, branched, hydrophobic leucine side chains into the P9 pocket within the I-A peptide-binding groove causes even greater steric strain at the binding interface than the native Met 99 CLIP residue. Indeed, Gautam *et al.* (1995) have examined previously the relative effects of the bulk of the Met 93 and Met 99 CLIP residues and shown that a progressive decrease in the number of methylene groups at these side-chain positions led to a significant improvement in the ability of this ligand to bind to the I-A^a, I-A^k and I-A^d molecules. However, Lee and coworkers still appear to have misinterpreted their data by taking such findings as confirmation that the wild-type methionine residues were anchors within the CLIP sequence for binding to I-A class II MHC molecules. A similar claim has been made more recently by Naujokas *et al.* (1998) upon observing a decrease in the affinity of the CLIP ligand for I-A^d when Met 91 was substituted with L-leucine. This is simply not supported by the previous experiments of Gautam *et al.* (1995) nor the results presented herein.

3.4.6 The effects of polymorphism within the class II MHC peptide-binding groove on the binding of CLIP

The experimental results summarised in Table 3.2 indicate that, although CLIP demonstrates promiscuous binding to many different class II MHC allotypes, the affinity for this ligand is by no means unaffected by the polymorphism of the I-A peptide-binding groove. This is illustrated similarly in the results of the cell-surface peptide binding assays by the different affinities of biotinylated CLIP86–104 for the three I-A variants (I-

A^d >> I-A^k > I-A^u; Figure 3.2). These findings are consistent with both the affinity measurements reported by Sette *et al.* (1995) and the different rates of CLIP dissociation from these molecules determined by Bangia & Watts (1995) and Liang *et al.* (1995), *e.g.* for CLIP86–102, K_d = 4.3 μM for I-A^d, >16 μM for I-A^k at pH 7.0.

Given that the affinity of a peptide for binding to a particular class II MHC molecule typically reflects the overall balance of attractive and repulsive forces present between the side chains of the peptide and the residues lining the groove (*Chapter 1*, §1.3.3), an explanation for the differing affinities of the I-A^d, I-A^k and I-A^u molecules for CLIP may be obtained by an examination of their respective binding motifs. Indeed, comparison of the X-ray crystallographic analysis of I-A^k (Fremont *et al.*, 1998b) and I-A^d (Scott *et al.*, 1998) reveals a significant difference between these molecules in the relative importance of their having the polymorphic pockets of the groove occupied by peptide side chains. For example, all of the five side chains of the HEL50–62 ligand that interact at these sites in the crystal structure of I-A^k were buried almost entirely, thereby contributing a large measure of positive binding energy to the complex (Fremont *et al.*, 1998b). By contrast, the X-ray crystallographic analysis of the I-A^d peptide-binding groove revealed the pockets in this molecule to be relatively shallow overall and frequently only partially filled by the side chains of the bound peptides, HA126–138 or Ova323–339 (Scott *et al.*, 1998).

It would thus appear that, unlike I-A^k molecules, ligands may bind well to I-A^d without having need of major anchor residues. Of particular note, the large P1 pocket in I-A^d appears able to accommodate peptide side chains of quite different physicochemical characteristics without necessarily having any effect upon the overall binding affinity (Bartnes *et al.*, 1997). This is in agreement with previous findings that this allotype exhibits a binding motif of only six consecutive amino acids (Sette *et al.*, 1987, 1989b; residues P4–P9, as determined by Scott *et al.*, 1998) instead of the usual nine-residue motif displayed by other class II MHC variants. Moreover, T cell recognition of such complexes does not appear to be strictly dependent upon the presence of a good anchor at the P1 position either (Bartnes *et al.*, 1997). Rather, the only key pocket interaction that does appear to be required for a peptide to bind to I-A^d involves the side chain at position P4. At this position, an alignment of known I-A^d-binding peptides and naturally-processed ligands has shown a critical preference for an aliphatic amino acid (Table 3.2; Bartnes *et al.*, 1997; Reizis *et al.*, 1998; Scott *et al.*, 1998). In both the crystal structures of I-A^d, this requirement is fulfilled by a valine residue (Val 131 in HA126–138, Val 327 in Ova323–339; Scott *et al.*, 1998).

It is therefore likely that CLIP is able to bind with high affinity to I-A^d class II MHC molecules because of their relatively unrestricted peptide-binding motif. By contrast, the CLIP sequence is less able to fulfil the more critical anchor requirements of the I-A^k molecule and, consequently, binds with diminished capacity. Following this argument, I-A^u would then be expected to exhibit the most restricted anchor requirements of all, given its very low affinity for the CLIP ligand (Figure 3.2). This latter idea is consistent with the recent finding that the very low affinity of the MBP *Ac*1-11 peptide for the I-A^u heterodimer arises from this ligand adopting a binding register which leaves part of the peptide-binding groove empty (the positions P1 and P2) and none of the polymorphic pockets accommodating a suitable, strong anchor side chain (Lee *et al.*, 1998). However, when a tyrosine residue was substituted into either the MBP *Ac*1–11 peptide or CLIP at a position corresponding with the large P6 pocket, a significant increase in the stability of binding was observed, as determined by measurement of the dissociation kinetics. A similar effect was obtained by extending the amino-terminus of the MBP *Ac*1–11 peptide with six residues of the NH_2-terminus of the Ova323–339 peptide. The ability of CLIP to bind to I-A^d, I-A^k and I-A^u allotypes without fulfilment of specific anchor requirements suggests that the interaction must therefore be driven by interactions through the peptide backbone. The contribution of the main-chain atoms of the CLIP sequence to the interaction with I-A class II MHC molecules is the subject of *Chapter 4*.

The effect of allelic variation in the modulation of the binding affinity of CLIP has also been reported for human class II MHC molecules (Avva & Cresswell, 1994; Sette *et al.*, 1995). For example, this ligand has been shown to bind with higher affinity to HLA-DR1 than to HLA-DR3 (Sette *et al.*, 1995). In this respect, it is likely that, similar to the situation for the I-A molecules, the differences seen in the CLIP binding affinities reflect the varying abilities of this sequence to fulfil the HLA-DR1 and DR3 binding motifs. For example, examination of the structure of the HLA-DR3 molecule and known peptide ligands reveals that, in addition to the preference for a large hydrophobic residue at the P1 position, there exists another major anchor requirement at the P4 pocket (Ghosh *et al.*, 1995; Rammensee *et al.*, 1995). Here, a strong preference is displayed for an aspartic acid side chain to interact with the HLA-DR3-specific arginine at position β74 (βAla 74 in HLA-DR1). In the interaction of CLIP with HLA-DR3 however, this position is occupied only by a small alanine residue (Ala 94) which leads to a slight collapse inwards in this region of the β-chain α-helix which forms one wall of the groove (Ghosh *et al.*, 1995). In the event of a non-ideal P4 anchor, as in the case of binding CLIP, HLA-DR3 molecules are then noted to show a secondary preference motif to compensate, in the form of a positively-charged residue at the P6 pocket to interact with the negatively-charged

βAsp 28 residue (Rammensee *et al.*, 1995). Again, this is not fulfilled when CLIP binds to HLA-DR3.

It is also likely that the overall superior ability of the CLIP sequence to meet the motif preferences of HLA-DR1 is the reason that this complex is able to attain stability in SDS detergent (Chicz *et al.*, 1992). Consistent with this argument, the introduction into the CLIP sequence of the HLA-DR3-specific anchor residue, aspartic acid, at the P4 position (Ala94Asp) also leads to the formation of an SDS-stable complex (Malcherek *et al.*, 1995) and these peptides are no longer able to be removed from the peptide-binding groove by the actions of HLA-DM (van Ham *et al.*, 1996). Conversely, replacement by an alanine residue of this P4 Asp anchor in the HLA-DR3-binding peptide, ApoB2877–2894, creates an SDS-unstable complex that is now susceptible to dissociation induced in the presence of HLA-DM. It has thus been suggested that the ability of HLA-DM to catalyse the removal of CLIP from class II MHC molecules may hinge upon this ligand lacking such optimal anchor residues and the resulting the stability they that confer (Kropshofer *et al.*, 1996; van Ham *et al.*, 1996; Weber *et al.*, 1996). It is worth noting, however, that the ability of a cognate peptide to confer resistance to the dissociation of the class II MHC subunits in the presence of SDS detergent is an arbitrary indicator. The molecular basis underlying this feature is not well understood and, frequently, the behaviour of a peptide–αβ complex upon SDS-PAGE does not correlate with the more relevant functional state of these molecules. For example, despite their SDS-stability, CLIP-bound HLA-DR1 molecules are still sensitive to the peptide-exchange functions of HLA-DM molecules (Sloan *et al.*, 1995; Kropshofer *et al.*, 1996). Moreover, the characteristics of a given peptide–class II MHC complex in SDS is unrelated to whether it may be recognised by $CD4^+$ T lymphocytes (Wu *et al.*, 1996). For this reason, one must be very cautious in using this property as a measure of how well a peptide binds to a given αβ dimer. The relationship between SDS-stability and peptide binding affinity is discussed further in *Chapter 6* (§6.4.4).

Lastly, but significantly, binding affinity is also affected by proton concentration. Low pH values approximating those found in the acidic intracellular compartments of metabolically-active APC have been shown to accelerate markedly the formation of functional antigen–class II MHC complexes (Jensen, 1990, 1991; Harding *et al.*, 1991; Wettstein *et al.*, 1991; Mouritsen *et al.*, 1992; Sette *et al.*, 1992b). In this study, the binding of both CLIP (Figure 3.4) and the ovalbumin peptide Ova322–339 (Figure 3.5a) to $I\text{-}A^d$ molecules was increased at pH 5.0 relative to binding at pH 7.0. The molecular processes underlying the ability of acidic conditions to enhance peptide binding to class II MHC molecules are discussed in *Chapter 5*, §5.4.4.

3.5. Conclusions

In this chapter, the contributions from individual side chains within the CLIP sequence to the binding interaction with the mouse class II MHC molecules, I-A^d, I-A^k and I-A^u have been examined. This has been achieved by using a set of CLIP analogues, each with a single L-alanine residue substitution introduced into the sequence, as competitors against biotinylated CLIP86–104 in either cell-surface binding assays at pH 7.0 or, for purified I-A^d, in an immunoadsorption assay at pH 5.0. The ability of such side chain-substituted peptides to bind to class II MHC I-A allotypes in these experiments was consistent with results obtained previously through their capacity to inhibit I-A-restricted T-cell responses (Gautam *et al.*, 1995).

The results presented herein, coupled with those of Gautam *et al.* (1995), indicate that the CLIP sequence binds to class II MHC molecules of the mouse I-A isotype without strong anchor residues. In this respect, the binding of CLIP differs from that of conventional antigen-derived ligands. Some measure of positive binding energy is provided to the association of CLIP with I-A through the side chains, Thr 95 and Met 102, together with Arg 92 for I-A^u and Met 91 for I-A^k. However, an examination of structural data of the I-A molecules (*e.g.* Lee & McConnell, 1995; Fremont *et al.*, 1998b; Scott *et al.*, 1998) indicates that these residues are not accommodated within the polymorphic pockets of the peptide-binding groove but, rather, contact the αβ dimer outside of this site where the sequence is more conserved. This is likely to facilitate the ability of the CLIP ligand to bind promiscuously to different class II MHC allelic variants.

Of the CLIP side chains that do bind within the pockets of the I-A peptide-binding groove, several are inhibitory to the interaction, *e.g.* Pro 96 at the P6 pocket and Met 99 at P9. These deleterious side-chain contacts, together with the lack of strong anchor residues, may be significant is ensuring that the CLIP ligand is able to be displaced efficiently *in vivo* to allow the binding and presentation of antigen-derived peptides. The lack of optimal anchor residues within the CLIP sequence suggests that the interaction must be maintained predominantly by another means, possibly *via* the backbone. The role of the main chain atoms of the CLIP backbone in the binding of this sequence to I-A molecules is investigated in *Chapter 4*.

CHAPTER 4.

The role of main-chain atoms in the binding of CLIP to mouse I-A class II MHC molecules

4.1. Introduction

In *Chapter 3*, the molecular basis of the promiscuous binding of CLIP to different class II MHC glycoproteins was explored by examining the contribution of the individual amino acid side chains within this peptide sequence. These experiments revealed that the substitution of most CLIP side chains had only minor effects upon the overall capacity of this ligand to bind to the mouse class II MHC allotypes, I-A^d, I-A^u and I-A^k. Moreover, the few native side chains that were seen to have a significant influence on the binding affinity of this interaction did so chiefly in an inhibitory manner.

If the amino acid side chains of the CLIP ligand do not form strong favourable contacts with the residues of the I-A $\alpha\beta$ dimers, then it follows that the interaction must instead be sustained predominantly by positive binding energy provided through the main-chain atoms of the peptide backbone. In this chapter, this possibility has been investigated by introducing stereochemical inversions into the CLIP86–104 sequence in the form of single D-alanine substitutions. Such modifications within a peptide typically lead to a breakdown of native secondary structure (DeGrado, 1988). It would be predicted, therefore, that the incorporation of a D-alanine residue within the CLIP ligand would also disrupt the capacity of its backbone to make contacts with the class II MHC heterodimer.

The contribution of main-chain interactions to the binding affinity of CLIP for the mouse allotypes, I-A^d, I-A^u and I-A^k, has been assessed using the D-alanine-substituted analogues in a series of competitive peptide-binding assays. Further details of the CLIP binding motif have been examined using truncated analogues, frameshifted 15-mers and additional L-alanine-substituted variants. The model proposed in *Chapter 3* to account for the degenerate binding of the CLIP sequence to class II MHC molecules has been extended here to incorporate these findings and the possibility is discussed that conserved residues within the amino-terminus of this ligand may also modulate its binding to different I-A molecules.

4.2. Materials and Methods

Full details of the experimental procedures described herein may be found in *Chapter 2*. Briefly, the mouse B cell lines, A20 (H-2^d), CH27 (H-2^k), M12.D (I-A^d) and 1-5.4 (I-A^u), were used as APC in both antigen presentation assays and cell-surface peptide

binding assays (Table 2.1). The I-A-restricted T cell hybridomas, 3DO-54.8 (I-A^d-restricted, Ova323–339-specific), 3A9 (I-A^k-restricted, HEL46–61-specific) and 1934.4 (I-A^u-restricted, MBP *Ac*1–11-specific), were used in the antigen presentation assays (Table 2.2), together with the IL-2 dependent T cell line, HT-2. For ELISA-based peptide binding assays, mouse I-A^d molecules were purified by Mrs Y. M. Gautam (Laboratory technician) from the BALB/c-derived B cell lymphoma, A20, by affinity chromatography on an MK-D6 mAb column (§2.6).

D-alanine-substituted CLIP86–104 peptides are listed in Table 4.1. Truncated and frameshifted 15-mer CLIP analogues are listed in Tables 4.2 and 4.3, respectively. Further substituted and length-altered CLIP analogues are given in Table 4.4. CLIP81–104 analogues with single L-alanine substitutions in the amino-terminus are shown in Table 4.5. The peptide concentrations used in binding assays are stated in the figure legends. Competitor peptides were tested over the range 3–200 μM in antigen presentation assays (§2.7.1), 32–250 μM for cell-surface binding assays (§2.7.2) and from 38–300 μM for immunoassays with purified I-A^d (§2.7.3).

The mouse invariant chain protein sequence used in the secondary structure predictions was retrieved from the integrated databases of the National Center for Biotechnology Information (NCBI) using the Entrez World Wide Web browser (http://www.ncbi.nlm.nih.gov/Entrez). Protein secondary structure predictions were conducted using two methods: the nearest neighbour secondary structure prediction service (NNSSP) of Salamov & Solovyev (1995) available from the University of Houston network server (service@theory.bchs.uh.edu; subject line "man nnssp" for help); and the method of Rost & Sander (1993, 1994) from the PHDsec web site (http://www.embl-heidelberg.de/predictprotein/predictprotein.html), accessed through the ExPASy proteomics server of the Swiss Institute of Bioinformatics (SIB; http://www.expasy.ch/tools/#secondary).

4.3. Results

4.3.1 D-alanine substitutions within the central CLIP sequence disrupt binding to I-A class II MHC molecules

Prior to their use in competitive binding assays, confirmation was first sought that the introduction of single D-alanine residues into the primary sequence of the CLIP86–104 ligand (Table 4.1) created the desired disruption of the secondary structure of the peptide.

Table 4.1 *D-alanine-substituted peptide analogues of CLIP86–104.*

K86D-Ala	D-Ala - - - - - - - - - - - - - - - - - -
P87D-Ala	- D-Ala - - - - - - - - - - - - - - - - -
V88D-Ala	- - D-Ala - - - - - - - - - - - - - - - -
S89D-Ala	- - - D-Ala - - - - - - - - - - - - - - -
Q90D-Ala	- - - - D-Ala - - - - - - - - - - - - - -
M91D-Ala	- - - - - D-Ala - - - - - - - - - - - - -
R92D-Ala	- - - - - - D-Ala - - - - - - - - - - - -
M93D-Ala	- - - - - - - D-Ala - - - - - - - - - - -
A94D-Ala	K P V S Q M R M D-Ala T P L L M R P M S M
T95D-Ala	- - - - - - - - - D-Ala - - - - - - - - -
P96D-Ala	- - - - - - - - - - D-Ala - - - - - - - -
L97D-Ala	- - - - - - - - - - - D-Ala - - - - - - -
L98D-Ala	- - - - - - - - - - - - D-Ala - - - - - -
M99D-Ala	- - - - - - - - - - - - - D-Ala - - - - -
R100D-Ala	- - - - - - - - - - - - - - D-Ala - - - -
P101D-Ala	- - - - - - - - - - - - - - - D-Ala - - -
M102D-Ala	- - - - - - - - - - - - - - - - D-Ala - -
S103D-Ala	- - - - - - - - - - - - - - - - - D-Ala -
M104D-Ala	- - - - - - - - - - - - - - - - - - D-Ala

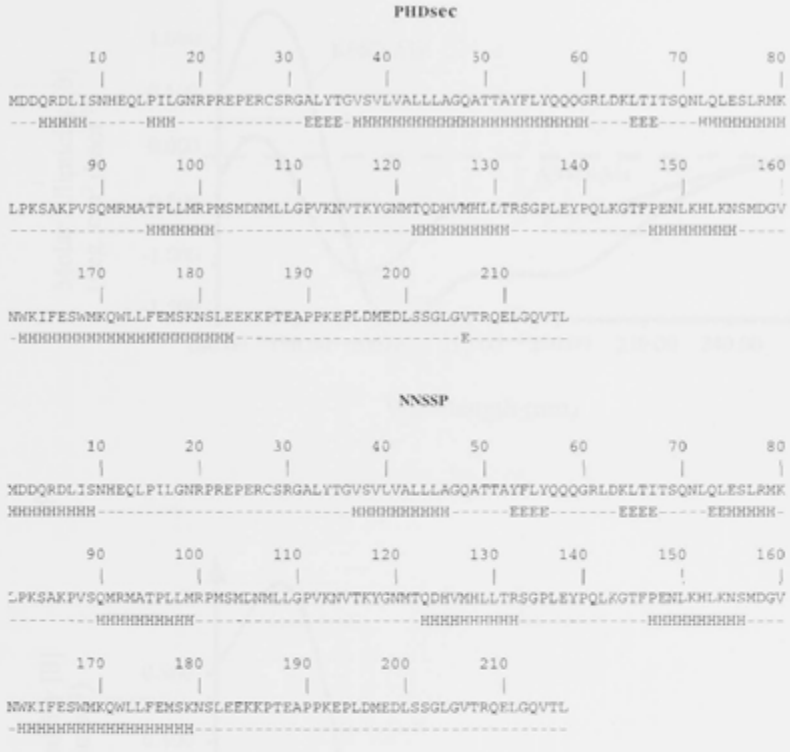
Amino acid sequences of CLIP86–104 analogues exhibiting single D-alanine substitutions are shown. Native amino acid residues are represented with a dash.

At the commencement of this investigation, the X-ray crystallographic analysis of the CLIP–HLA-DR3 complex had yet to be published and, therefore, details of the manner in which the CLIP backbone may interact with the class II MHC molecule were unknown (Ghosh *et al.*, 1995). However, an estimate of the potential conformation favoured by the CLIP ligand could be obtained using secondary structure prediction algorithms. Analysis by two such methods revealed that the wild-type CLIP sequence between residues 90–101 exhibited strong helix-forming potential (Figure 4.1). Moreover, this conformation was preferred irrespective of whether the entire sequence of intact mouse p31 invariant chain was analysed (Figure 4.1a) or just the region of this protein encoded by exon three, the sequence CLIP81–107 (Figure 4.1b).

To determine the disruptive effects of the D-alanine substitutions upon this possible conformation of the CLIP sequence, the circular dichroism (CD) of each of the configurationally-substituted CLIP analogues was measured under experimental conditions that strongly promoted the formation of α-helices. Shown in Figure 4.2a are the CD spectra of the monosubstituted CLIP86–104 variants, K86D-Ala and A94D-Ala, representative of the two extremes in conformation found amongst the D-alanine-substituted CLIP. Specifically, the CLIP analogues that exhibited D-alanine monosubstitutions at the peptide terminus (*e.g.* K86D-Ala) exhibited similar CD spectra to the wild-type peptide, CLIP86–104, while the introduction of a single D-alanine residue within the central region of this sequence caused a pronounced disruption of the native peptide conformation (*e.g.* A94D-Ala). Under the extreme α-helix-promoting conditions employed, the difference CD of these two peptides (Figure 4.2b) revealed a marked weakening of features characteristic of α-helical conformation in the A94D-Ala peptide, *i.e.* the loss of minima at 208 nm and 222 nm (Johnson, 1990). If the introduction of a D-alanine residue is sufficient to destroy the formation of an α-helix within this peptide under these conditions then, by extension, such substitutions may also be expected to disrupt any other form of periodic conformational structure that the CLIP ligand may potentially adopt.

The role of the CLIP backbone in its binding to I-A class II MHC molecules was then examined using the D-alanine-substituted analogues as competitors in antigen presentation assays with the same APC lines and I-A-restricted T cell hybridomas as employed previously to investigate the binding of the L-alanine-substituted CLIP analogues (Gautam *et al.*, 1995). In contrast to the findings with the side chain-substituted analogues, the introduction of a single D-alanine amino acid into the central region of the peptide between residues, Met 91 and Met 99, was found to eliminate or reduce

a. mouse p31 invariant chain



b. mouse CLIP81–107

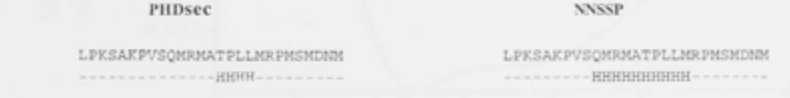


Figure 4.1 *Secondary structure prediction of mouse invariant chain p31 and CLIP81–107.*

Conformational tendencies of **(a)** mouse invariant chain, p31, and **(b)** the CLIP sequence 81–107 were examined using different secondary structure prediction algorithms. Shown are the results of two such methods (PHDsec: Rost & Sander, 1993, 1994; NNSSP: Salamov & Solovyev, 1995) as described in *Material and Methods* (§4.2). Secondary structure predictions are indicated by H (helix) and E (strand). A dash indicates no statistically-preferred conformation. Mouse p31 Ii is numbered according to the human p33 sequence in accordance with the numbering used throughout this thesis (*Chapter 1*, Figure 1.5). The mouse Ii sequence bears two deletions at postions 27 and 138 and an insertion at position 185a relative to the human Ii sequence.

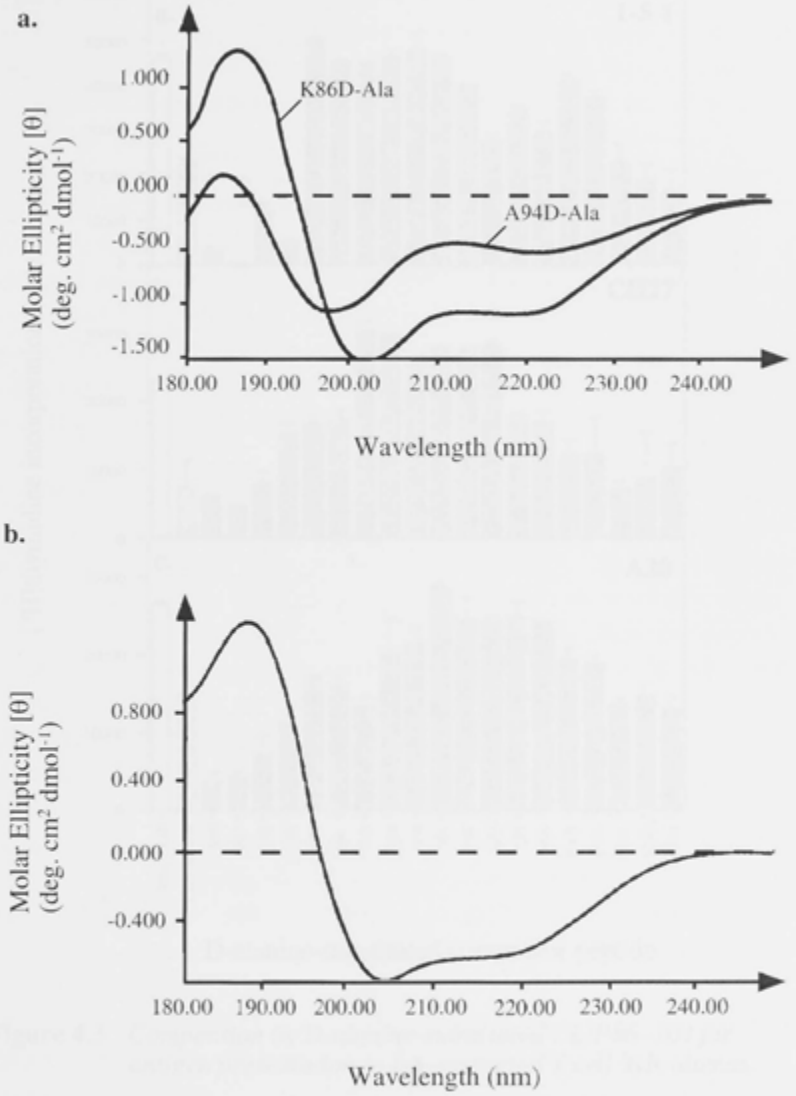


Figure 4.2 Circular dichroism of D-alanine-substituted CLIP86–104.

(a) Circular dichroic (CD) spectra of the configurationally-substituted CLIP86–104 analogues, K86D-Ala and A94D-Ala were recorded under strong α-helix-promoting conditions in 50% (v/v) TFE/ 10 mM sodium phosphate, pH 7.0 at 25°C, as described in *Chapter 2*, §2.3.3. The difference CD between these peptides **(b)** shows abrogation of helix-forming propensity in the A94D-Ala CLIP analogue.

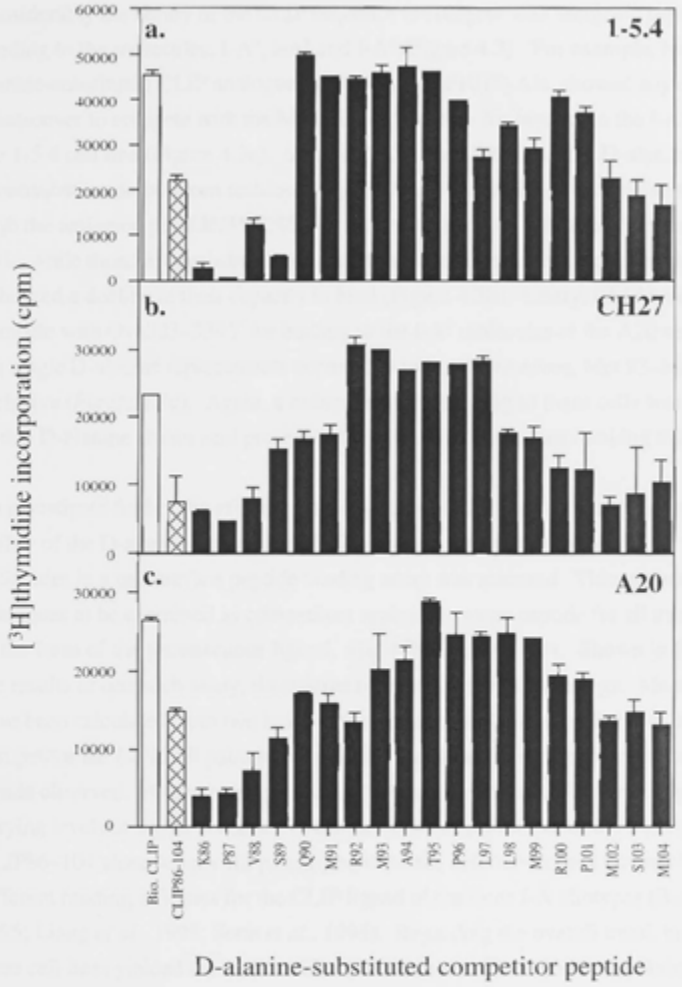


Figure 4.3 *Competition by D-alanine-substituted CLIP86–104 for antigen presentation to I-A-restricted T cell hybridomas.*

Multiple doses of CLIP86–104 analogues with single D-alanine substitutions were tested in triplicate as competitors for presentation by the cell lines **(a)** 1-5.4 (I-A^s), **(b)** CH27 (H-2^k) and **(c)** A20 (H-2^d) to the I-A-restricted T cell hybridomas, 1934.4 (MBP *Ac*1–11, 5 μM), 3A9 (HEL46-61, 1.5 μM) and 3DO-54.8 (Ova323–339Y, 0.05 μM), respectively. A single competitor dose which yielded ~50% mean inhibition of antigenic T cell stimulation by wild-type CLIP86–104 (cross-hatched fill pattern) is shown from one experiment (1-5.4, 50 μM; CH27, 100 μM; A20, 12.5 μM). Solid bars represent mean competition by the substituted CLIP analogues and empty bars indicate maximal T cell stimulation in the absence of competitor peptide. All experiments were repeated three times with consistent results.

considerably the ability of the CLIP sequence to compete with antigenic peptide for binding to the molecules, I-A^u, I-A^k and I-A^d (Figure 4.3). For example, few of the D-alanine-substituted CLIP analogues, Q90D-Ala to P101D-Ala, showed any capacity whatsoever to compete with the MBP *Ac*1–11 peptide for binding to the I-A^u molecules of the 1-5.4 cell line (Figure 4.3a). Similarly, CLIP analogues with a D-alanine monosubstitution between residues, Arg 92–Leu 97 inclusive, were unable to compete with the antigenic peptide, HEL46–61, for presentation by I-A^k molecules of the CH27 cells, while those with substitutions immediately adjacent to this sequence element exhibited a decline in their capacity to bind (Figure 4.3b). Lastly, CLIP analogues did not compete with Ova323–339Y for binding to the I-A^d molecules of the A20 cell line when the single D-alanine replacements occurred between the residues, Met 93–Met 99 inclusive (Figure 4.3c). Again, a reduction in the binding to these cells was observed with a D-alanine amino acid present in the two or three residues flanking this.

To investigate further the effects of modifying the CLIP backbone stereochemistry, the ability of the D-alanine-substituted CLIP analogues to bind to the I-A^u, I-A^k and I-A^d molecules in a cell-surface peptide binding assay was assessed. This method enables the analogues to be examined as competitors against the same peptide for all three allotypes, in the form of the promiscuous ligand, wild-type CLIP86–104. Shown in Figure 4.4 are the results of one such assay, illustrative of the experimental findings. Mean IC_{50} values have been calculated from two independent experiments and are given atop each competitor bar (solid fill pattern) to provide a quantitative representation of the overall trends observed. As observed previously using this method (*Chapter 3*, Figure 3.2), the varying levels of signal generated in the positive control sample of the biotinylated CLIP86–104 alone (empty fill pattern) between the cell lines reflect accurately the different binding affinities for the CLIP ligand of the three I-A allotypes (Bangia & Watts, 1995; Liang *et al.*, 1995; Sette *et al.*, 1995). Regarding the overall trend, however, all three cell lines yielded the same result. A considerable reduction in the ability of CLIP to compete for cell-surface binding was observed when the D-alanine residues were introduced into the core of the sequence. This finding is consistent with those from the antigen presentation assays performed with the same cell lines (Figure 4.3).

In *Chapter 3* (§3.3.1), the possibility that the results from cell-surface binding assays performed with the cell lines CH27 and A20 may also include a contribution from CLIP associating with I-E class II MHC molecules was discussed. This was deemed to be unlikely given the similarity in the pattern of results between the binding of the L-alanine-substituted CLIP to the cell-surface class II MHC molecules (*Chapter 3*, Figure 3.2) and

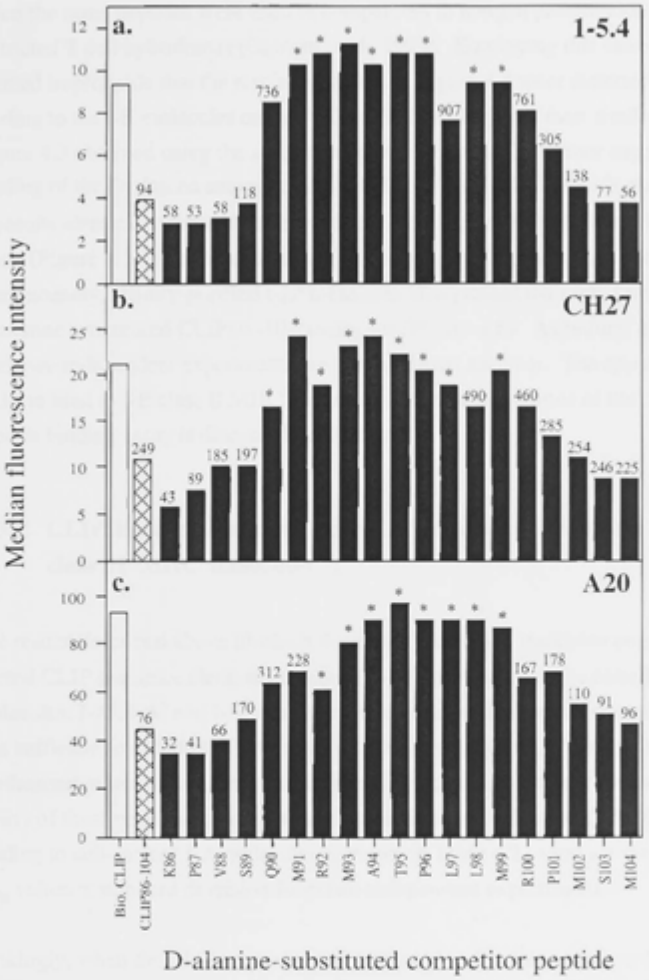


Figure 4.4 *Competition by D-alanine-substituted CLIP86–104 for binding to cell-surface I-A class II MHC molecules.*

Multiple doses of CLIP86–104 analogues with single D-alanine substitutions were tested for competition against biotinylated CLIP86–104 (20 μM) for binding to cell-surface I-A molecules of the cell lines (**a**) 1-5.4 (I-A^{u}), (**b**) CH27 (I-A^{k}) and (**c**) A20 (I-A^{d}). A single competitor dose yielding ~50% inhibition of biotinylated CLIP binding by wild-type CLIP86–104 (cross-hatched fill pattern) is shown (1-5.4, 125 μM; CH27, 200 μM; A20, 62.5 μM). Assays were performed three times with consistent results. Solid bars represent competition by substituted CLIP and empty bars represent the maximal biotinylated CLIP signal with no competitor present. Binding levels are expressed as median fluorescence intensity (MFI), from which background counts have been subtracted (1-5.4, 3.05; CH27, 3.28; and A20, 3.05). IC_{50} values for each competitor peptide are given on top of each bar. An asterisk represents an IC_{50} value of >1000 μM.

when the same peptides were used as competitors in antigen presentation assays with I-A-restricted T cell hybridomas (Gautam *et al.*, 1995). Employing this same logic, it also seemed improbable that the results presented in Figure 4.4 were distorted by CLIP binding to the I-E molecules on the A20 or CH27 cells, given their similarity to those in Figure 4.3 obtained using the antigen presentation assay. In further support of this, the binding of the D-alanine analogues to the I-E° cell line, M12.D (I-A^d), showed a pattern of results identical to those obtained with the A20 cell line in both the antigen presenting assay (Figure 4.5a) and the cell-surface binding assay (Figure 4.5b). Moreover, in an immunoassay, affinity-purified I-A^d molecules also yielded the same trend for binding the D-alanine substituted CLIP86–104 analogues (Figure 4.6). As before, mean IC_{50} values from two independent experiments are provided atop each bar. The apparent inability of CLIP to bind to I-E class II MHC molecules under the conditions of the cell-surface peptide binding assay is discussed in *Chapter 5*.

4.3.2 CLIP ligands truncated below 13 residues bind poorly to I-A class II MHC molecules

The results described above illustrate the importance of the backbone conformation in the central CLIP sequence element, residues 91–99, for binding to the class II MHC molecules, I-A^s, I-A^k and I-A^d. In order to determine whether this central motif alone was sufficient to uphold this interaction, a set of truncated CLIP analogues was synthesised in which residues were sequentially deleted pairwise from each end. The ability of these peptides to compete against biotinylated wild-type CLIP 86–104 for binding to cell-surface I-A molecules is shown in Table 4.2. Data are presented as mean IC_{50} values ± standard deviation from two independent experiments.

Strikingly, when the CLIP sequence was truncated to 13 amino acids or fewer, its ability to compete for binding to all three allotypes was reduced (*e.g.* CLIP89–101, 90–100; Table 4.2) or even eliminated (*e.g.* CLIP91–99). An equivalent result was obtained using the truncated CLIP as competitors in antigen presentation assays (Figure 4.7). The central CLIP sequence element, residues 91–99, is therefore insufficient on its own to sustain binding to I-A class II MHC molecules.

The binding of the truncated CLIP was subject to some allele-specific effects, as observed previously with the D-alanine-substituted analogues (Figures 4.3 & 4.4). In particular, in

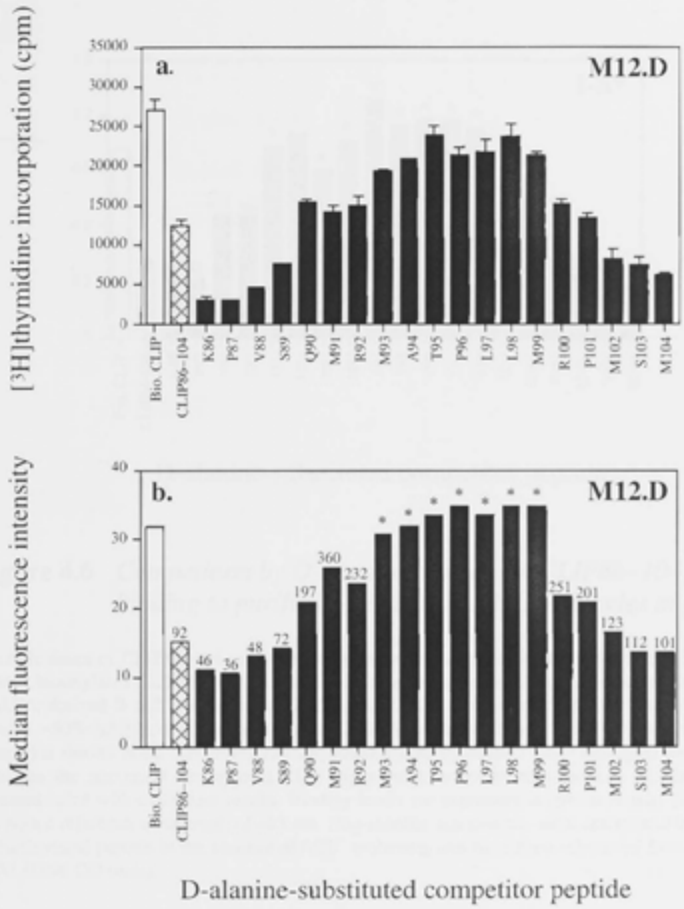


Figure 4.5 *Competition by D-alanine-substituted CLIP86–104 for binding to M12.D at pH 7.0.*

Multiple doses of CLIP86–104 analogues with single D-alanine substitutions were tested as competitiors for binding to I-A^d class II MHC molecules expressed by the cell line, M12.D, against **(a)** Ova323–339Y (0.05 μM) in an antigen presenting assay with the I-A^d-restricted T cell hybridoma, 3DO-54.8, and **(b)** biotinylated CLIP86–104 (20 μM) in a cell-surface binding assay. Shown is the dose which yielded ~50% mean inhibition of antigenic T cell stimulation from triplicate samples (12.5 μM) or ~50% inhibition of the binding of biotinylated CLIP (62.5 μM) by wild-type CLIP86–104 (cross-hatched fill pattern), respectively. Both antigen presentation assays and cell-surface peptide binding assays were each performed twice with consistent results. Solid bars represent competition by the substituted CLIP analogues. For each assay, the maximum signal with no competitor present is depicted by the empty bar. Background signals measured in the absence of signal peptides have been subtracted from data shown (**a**, 199 cpm; **b**, MFI, 2.46).

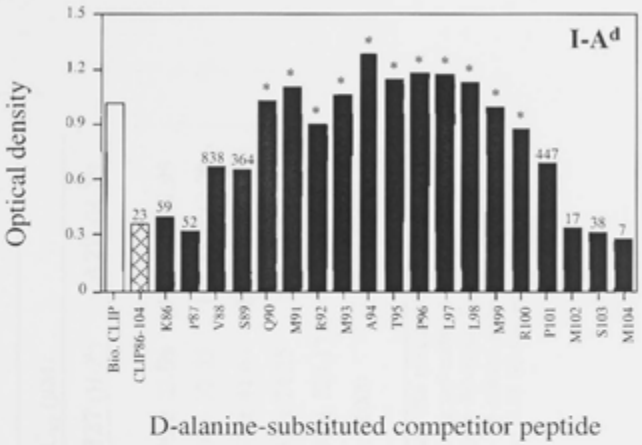


Figure 4.6 *Competition by D-alanine-substituted CLIP86–104 for binding to purified I-A^d class II MHC molecules at pH 5.0.*

Multiple doses of CLIP86–104 analogues with single D-alanine substitutions were tested as competitors against biotinylated CLIP86–104 (25 μM) for binding to I-A^d class II MHC molecules purified from the BALB/c-derived B cell lymphoma, A20 (*Chapter 2*, §2.6). A single competitor dose (150 μM) which yielded ~50% inhibition of the biotinylated CLIP signal by wild-type CLIP86–104 (cross-hatched fill pattern) is shown. Solid bars represent competition by the substituted CLIP analogues and the empty bar indicates the maximum biotinylated CLIP signal with no competitor present. Binding assays were repeated twice with equivalent results. Binding levels are expressed as optical density (OD) read at 405 nm with a reference wavelength of 495 nm. Non-specific associations were determined from the binding of biotinylated peptide in the absence of MHC molecules and have been subtracted from the data shown (I-A^d, 0.096 OD units).

Table 4.2 *Competition by truncated CLIP for binding to I-A class II MHC molecules.*

CLIP		IC_{50} (μM)		
		1-5.4 (I-A^u)	**CH27** (H-2^k)	**A20** (H-2^d)
86–104	KPVSQMRMATPLLMRPMSM	192.95 ± 22.27	309.30 ± 22.06	123.95 ± 11.95
87–103	PVSQMRMATPLLMRPMS	52.99 ± 4.07	149.80 ± 32.53	141.45 ± 12.09
88–102	VSQMRMATPLLMRPM	112.70 ± 7.50	143.55 ± 41.65	202.95 ± 3.18
89–101	SQMRMATPLLMRP	583.05 ± 139.80	429.35 ± 24.25	649.40 ± 318.20
90–100	QMRMATPLLMR	> 1000	842.90 ± 222.17	> 1000
91–99	MRMATPLLM	> 1000	> 1000	> 1000

Residues were removed pairwise, sequentially from each end of the CLIP86–104 sequence. The truncated CLIP were tested at multiple doses as competitors against biotinylated CLIP86–104 (50 μM) for binding to the cell-surface I-A molecules of the cell lines, 1-5.4, CH27 and A20, at pH 7.0. Data are presented as mean IC_{50} values ± SD (μM) from two independent experiments. Background median fluorescence intensity (MFI) values of 3.54, 5.14 and 3.6 relative units were recorded for I-A^u, I-A^k and I-A^d, respectively. The maximum MFI for each allotype was 13.6, 48.8 and 15.15 relative units, respectively.

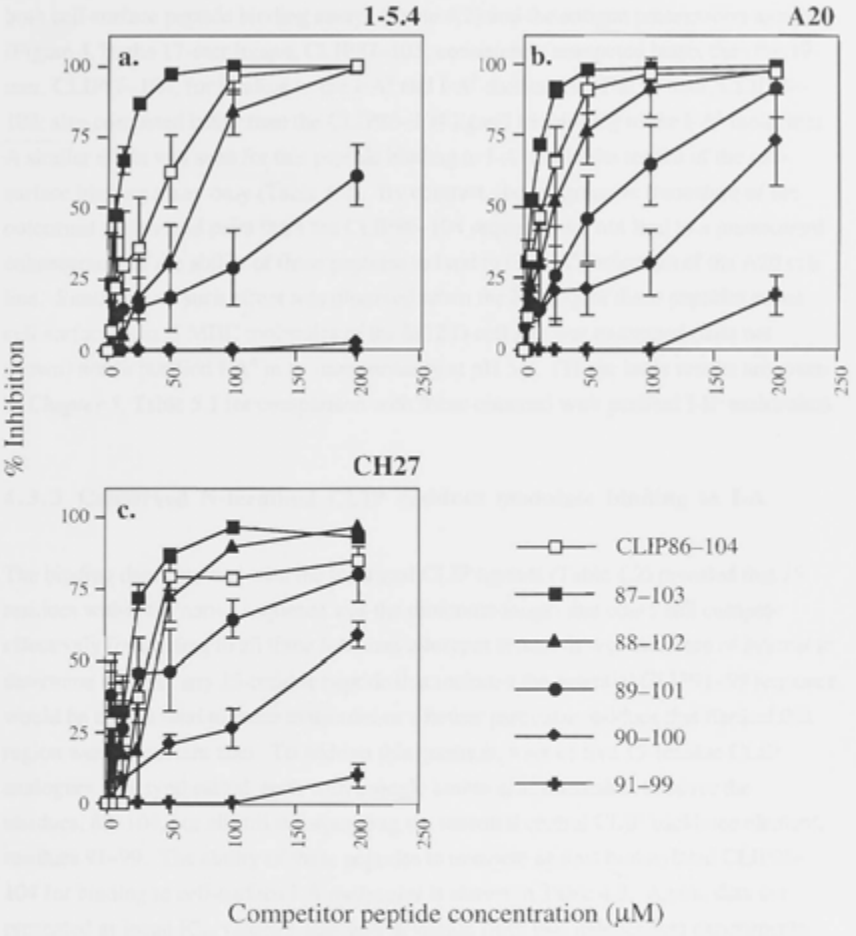


Figure 4.7 *Competition by truncated CLIP ligands for binding to I-A class II MHC molecules at pH 7.0.*

Truncated CLIP analogues from which residues were removed pairwise from each end were tested as competitors for presentation by the cell lines **(a)** 1-5.4 (I-A^u), **(b)** A20 (H-2^d) and **(c)** CH27 (H-2^k) to the I-A-restricted T cell hybridomas, 1934.4 (MBP *Ac*1–11, 5 μM), 3DO-54.8 (Ova323–339Y, 0.05 μM) and 3A9 (HEL46–61, 1.5 μM), respectively. Data shown represent the mean percentage inhibition ± SD from two independent experiments. Within each experiment, peptides were tested in triplicate samples, as described in *Chapter 2*, §2.7.1.

both cell-surface peptide binding assays (Table 4.2) and the antigen presentation assays (Figure 4.7), the 17-mer ligand, CLIP87–103, consistently competed better than the 19-mer, CLIP86–104, for binding to the I-A^u and I-A^k molecules. The 15-mer, CLIP88–102, also competed better than the CLIP86–104 ligand for binding to the I-A^k molecules. A similar effect was seen for this peptide binding to I-A^u but in the results of the cell-surface binding assay only (Table 4.2). By contrast, the progressive truncation of the outermost amino acid pairs from the CLIP86–104 sequence did not lead to a pronounced enhancement of the ability of these peptides to bind to the I-A^d molecules of the A20 cell line. Similarly, no such effect was observed when the binding of these peptides to the cell-surface class II MHC molecules of the M12.D cell line was examined (data not shown) nor to purified I-A^d in an immunoassay at pH 5.0. (These latter results are given in *Chapter 5*, Table 5.1 for comparison with those obtained with purified I-E^d molecules).

4.3.3 Conserved N-terminal CLIP residues modulate binding to I-A

The binding data obtained with the truncated CLIP ligands (Table 4.2) revealed that 15 residues within the native sequence was the minimum length that could still compete effectively for binding to all three I-A class allotypes tested. It was therefore of interest to determine whether any 15-residue peptide that included the essential CLIP91–99 sequence would be able to bind to these molecules or whether particular residues that flanked this region were important also. To address this question, a set of five 15-residue CLIP analogues was synthesised, each with a single amino acid frameshift to cover the residues, 86–104, but all still incorporating the essential central CLIP backbone element, residues 91–99. The ability of these peptides to compete against biotinylated CLIP86–104 for binding to cell-surface I-A molecules is shown in Table 4.3. Again, data are presented as mean IC_{50} values ± standard deviation from two independent experiments.

Overall, those CLIP ligands that contained all or part of the original N-terminal sequence (CLIP86–100, 87–101 and CLIP88–102) were found consistently to compete better for binding to the I-A molecules than those that exhibited the corresponding C-terminal CLIP residues (CLIP89–103, 90–104). As with the truncated peptides (Table 4.2), it was also apparent that the loss of the 1–2 outermost amino-terminal residues again improved the binding capacity to I-A^u and I-A^k, provided at least two residues were still present adjacent to the central 91–99 core sequence. Once more, this effect did not extend to the binding of these ligands to the I-A^d molecules of A20, nor to those of the M12.D cell line

Table 4.3 *Competition by frameshifted CLIP 15-mers for binding to I-A class II MHC molecules.*

CLIP		IC_{50} (μM)		
		1-5.4 (I-A^u)	**CH27** (H-2^k)	**A20** (H-2^d)
86–100	KPVSQMRMATPLLMR	183.05 ± 21.14	238.20 ± 25.14	197.45 ± 4.17
87–101	PVSQMRMATPLLMRP	62.62 ± 7.51	188.20 ± 21.74	157.65 ± 18.46
88–102	VSQMRMATPLLMRPM	112.70 ± 7.50	143.55 ± 41.65	202.95 ± 3.18
89–103	SQMRMATPLLMRPMS	> 1000	586.70 ± 184.98	437.45 ± 33.59
90–104	QMRMATPLLMRPMSM	> 1000	> 1000	576.40 ± 180.60

Fifteen-residue CLIP analogues were synthesised, each with a with a single amino acid frameshift. Analogues were tested at multiple doses as competitors against biotinylated CLIP86–104 (50 μM) in a cell-surface binding assay at pH 7.0. Data are presented as mean IC_{50} values ± SD (μM) from two independent experiments. Background levels and maximum signals of median fluorescence intensity (MFI) for each allotype are as in Table 4.2.

in a cell-surface binding assay (data not shown) or to purified I-A^d in an immunoassay at pH 5.0 (Shown in *Chapter 5*, Table 5.2 for comparison with purified I-E^d).

The contribution of the particular amino acid residues that flank the central sequence element, CLIP91–99, to the interaction with the molecules, I-A^u, I-A^k and I-A^d, was explored further by assessing the binding abilities of another set of CLIP analogues, this time exhibiting a number of different truncations and/or L-alanine substitutions. Shown in Table 4.4 are the results of these peptides competing against biotinylated CLIP86–104 for binding to cell-surface I-A molecules. Data are presented as mean IC_{50} values ± standard deviation from two independent experiments. Some improvement in the binding affinity of the CLIP sequence for I-A^k molecules was apparent when the C-terminal residue, Met 104, was deleted. However, of most interest, the binding to this allotype was again sensitive to modifications of the two outermost amino-terminal residues of the CLIP86–104 sequence, Lys 86 and Pro 87. For example, substitution of either or both of these residues with L-alanine enhanced the ability of the resultant CLIP analogue to bind (*e.g.* Ala-CLIP, CLIP87A–104). Similarly, truncation of these residues enhanced the binding capacity of CLIP (*e.g.* 88–103). This time, however, these effects were not as pronounced for the binding of this sequence to class II MHC molecules of the I-A^u allotype, except in the case of the truncated peptide, CLIP87–102. Moreover, consistent with previous findings (Tables 4.2 & 4.3), no such manipulations of the CLIP sequence improved the binding of this ligand to the cell-surface I-A^d molecules of the A20 cell line (Table 4.4) nor to purified I-A^d in an immunoassay at pH 5.0 (*Chapter 5*, Table 5.3).

The findings described above concerning the ability of the outermost N-terminal residues to influence the binding affinity of this peptide sequence for certain I-A allotypes were particularly interesting in light of a report published subsequently suggesting that the N-terminal residues of the human CLIP sequence may provide a "self-release" mechanism by which this ligand could facilitate its own dissociation from HLA-DR molecules independently of the actions of HLA-DM (Kropshofer *et al.*, 1995a, 1995b). In order to investigate whether such a mechanism was also operative in the interaction of CLIP with mouse I-A molecules, peptides exhibiting single L-alanine substitutions in the amino-terminal region of the CLIP81–104 sequence (residues 81–87) were synthesised (Table 4.5) and examined once more for binding to the different I-A-expressing cell lines.

The titration of biotinylated forms of the N-terminal-substituted mouse CLIP analogues with A20 cells in a cell-surface binding assay (Figure 4.8) revealed that substitution of several of the individual native N-terminal residues did indeed lead to a change in the

Table 4.4 *Competition by substituted and/or length-altered CLIP for binding to I-A class II MHC molecules.*

CLIP		IC_{50} (μM)		
		1-5.4 (I-A^u)	**CH27** (H-2^k)	**A20** (H-2^d)
86–104	KPVSQMRMATPLLMRPMSM	192.95 ± 22.27	309.30 ± 22.06	123.95 ± 11.95
Ala-CLIP	AAVSQMRMATPLLMRPMAA	151.35 ± 37.83	168.05 ± 35.85	161.85 ± 2.05
86–103	KPVSQMRMATPLLMRPMS	181.70 ± 3.82	221.75 ± 39.39	178.25 ± 5.30
86–103A	KPVSQMRMATPLLMRPMA	180.15 ± 6.01	220.80 ± 9.62	172.55 ± 0.78
87–104	PVSQMRMATPLLMRPMSM	126.95 ± 5.87	309.45 ± 43.20	128.95 ± 5.87
87A–104	AVSQMRMATPLLMRPMSM	192.35 ± 13.22	241.35 ± 38.68	138.85 ± 9.55
87–102	PVSQMRMATPLLMRPM	70.92 ± 21.49	337.30 ± 174.37	132.90 ± 18.10
88–103	VSQMRMATPLLMRPMS	165.55 ± 0.64	215.30 ± 46.10	187.00 ± 14.57

Substituted and/or length-altered CLIP analogues were tested at multiple doses as competitors against biotinylated wild-type CLIP86–104 (50 μM) for binding to the cell-surface I-A class II MHC molecules of the cell lines, 1-5.4, CH27 and A20. Data are presented as mean IC_{50} values ± SD (μM) from two independent experiments. Background levels and maximum signals of median fluorescence intensity (MFI) for each allotype are as in Table 4.2.

Table 4.5 *N-terminal L-alanine-substituted CLIP81–104 analogues*

L81A	L-Ala -
P82A	- L-Ala -
K83A	- - L-Ala -
S84A	- - - L-Ala - - - - - - - - - - - - - - - - - - -
wild-type	L P K S L-Ala K P V S Q M R M A T P L L M R P M S M
K86A	- - - - - L-Ala - - - - - - - - - - - - - - - - -
P87A	- - - - - - L-Ala - - - - - - - - - - - - - - - -

Amino acid sequences of CLIP81–104 analogues bearing single L-alanine substitutions within their N-terminus, residues 81–87. Native amino acid residues are represented with a dash.

ability of these peptides to bind to the I-A^d molecules expressed by this cell line. However, rather than decreasing the ability of CLIP to binding to this MHC molecule, the native residues within this region of the ligand appeared to enhance binding. Specifically, when any one of the CLIP residues, Lys 83, Lys 86 or Pro 87, was replaced by L-alanine, a marked decline in the binding capacity was observed relative to that of the wild-type CLIP81–104. A similar result was obtained when these same peptides were tested for binding to the cell-surface I-A^d molecules of the M12.D cell line (Figure 4.9a) and to purified I-A^d in an immunoassay at pH 5.0 (Figure 4.9b). These findings are consistent with those obtained with the various truncated, frameshifted and substituted peptides binding to A20 cells shown in Tables 4.2–4.4. For example, modifications of the outermost N-terminal residues of the CLIP86–104 element, such as those seen in the peptides, Ala-CLIP and CLIP88–103, also caused a decrease in the capacity of this sequence to bind to this class II MHC allotype (Table 4.4). Furthermore, binding to these molecules was not affected noticeably by similar substitutions of single L-alanine amino acid residues into the shorter CLIP86–104 ligand in *Chapter 3* (Figures 3.2 & 3.3).

It was therefore of interest to examine the interaction of the N-terminal L-alanine-substituted CLIP81–104 analogues with the mouse class II MHC allotypes, I-A^u and I-A^k, which had displayed previously an increase in their affinity for this ligand upon modification of its N-terminal residues (Tables 4.2–4.4). Titration of the biotinylated N-terminal-substituted CLIP with the cell lines, 1-5.4 (I-A^u) and CH27 (H-2^k) is shown in Figure 4.10. Surprisingly, the binding of these peptides to I-A^u molecules (Figure 4.10a) yielded a similar, although less prominent, pattern of results to that obtained with the I-A^d

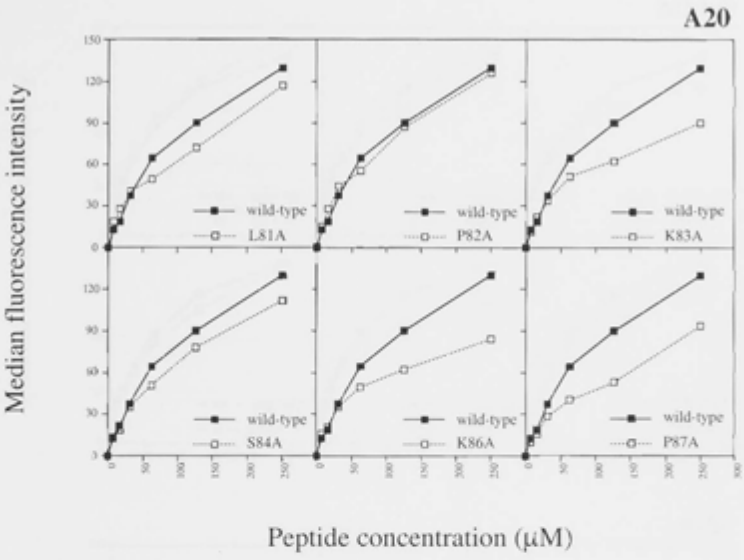


Figure 4.8 *The binding of N-terminal L-alanine-substituted CLIP81–104 analogues to A20 cells at pH 7.0.*

Biotinylated CLIP81–104 analogues with single L-alanine substitutions in their N-terminus (dashed lines, open squares) were compared with biotinylated wild-type CLIP81–104 (solid line, filled squares) for cell-surface binding to the A20 cell line (H-2^d). Shown is one of five independent experiments, all yielding equivalent results. Binding levels are expressed as median fluorescence intensity (MFI). The background signal was determined in the absence of biotinylated peptide and has been subtracted from data shown (A20, 3.28). Cells were incubated with biotinylated peptide for 18 hours at 37°C before flow cytometric analysis, as described in *Chapter 2*, §2.7.2.

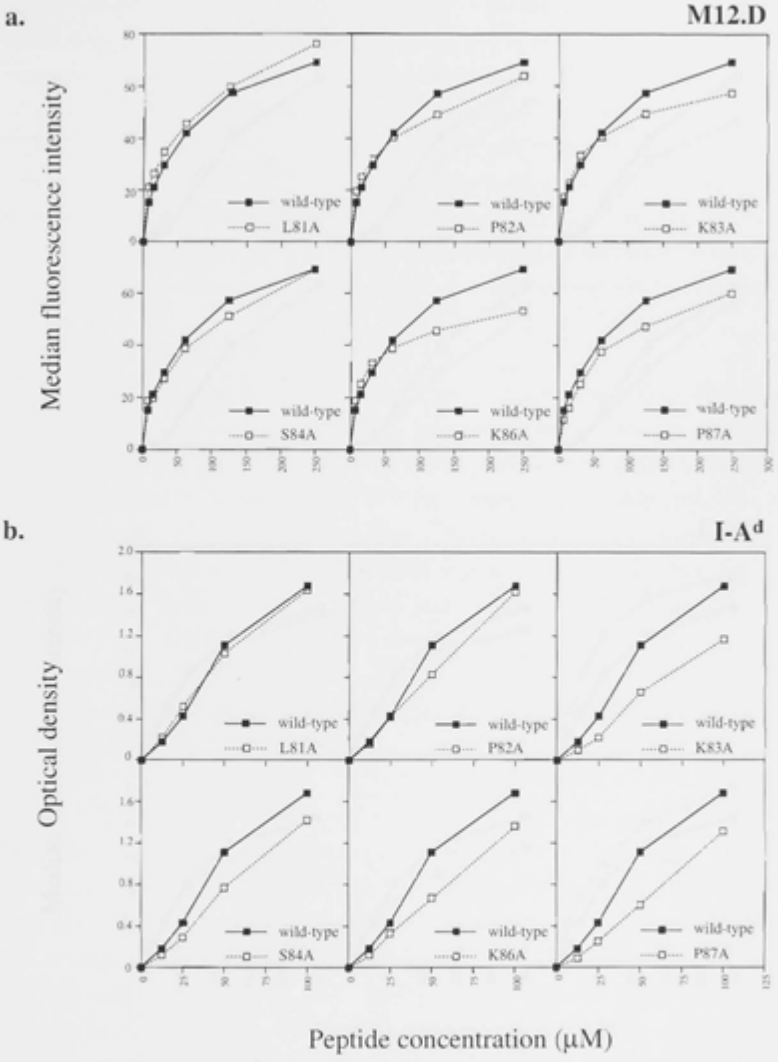


Figure 4.9 *The binding of N-terminal-substituted CLIP81–104 to M12.D cells and purified I-A^d class II MHC molecules.*

Biotinylated CLIP81–104 analogues with single L-alanine substitutions in their N-terminus (dashed lines, open squares) were compared with biotinylated, wild-type CLIP81–104 (solid line, filled squares) for binding to **(a)** the cell-surface I-A^d molecules of the M12.D cell line at pH 7.0 and **(b)** purified I-A^d proteins at pH 5.0. Experiments were each performed three times, as described in *Chapter 2*, §2.7.2 & §2.7.3. Background signal was determined in the absence of biotinylated peptide and has been subtracted from the data shown (M12.D, 3.28; I-A^d, 0.052 OD units).

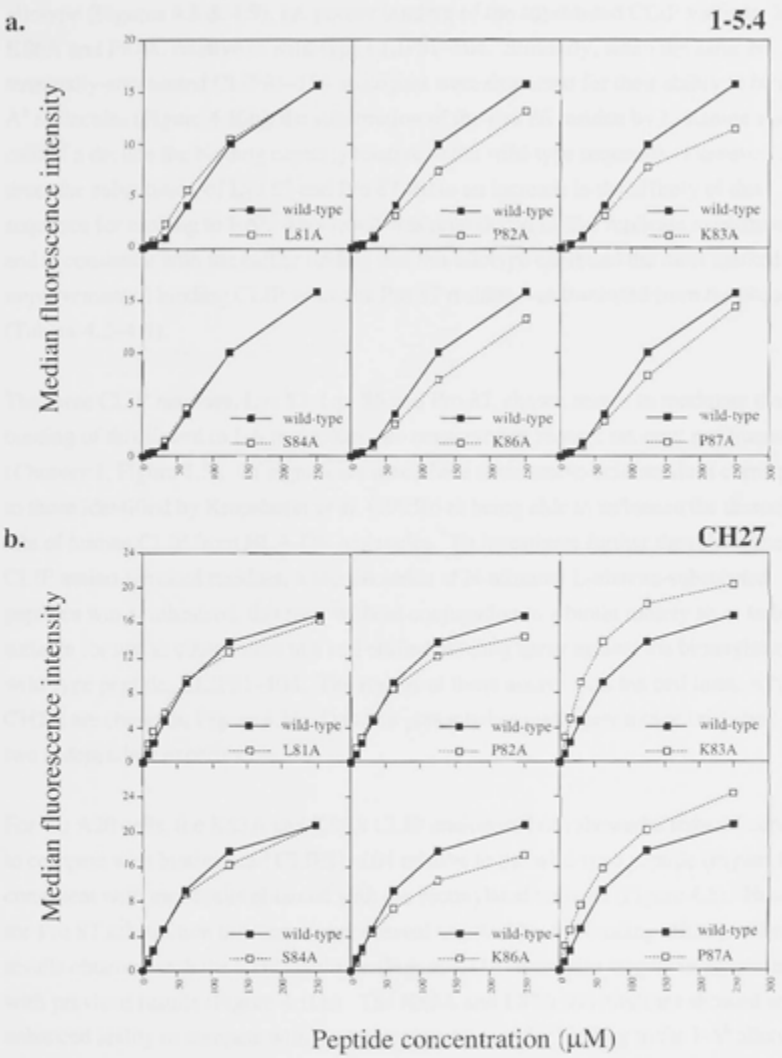


Figure 4.10 *The binding of N-terminal-substituted CLIP81–104 to the cell lines, 1-5.4 and CH27 at pH 7.0.*

Biotinylated CLIP81–104 analogues with single L-alanine substitutions in their N-terminus (dashed lines, open squares) were compared with wild-type biotinylated CLIP81–104 (solid line, filled squares) for binding to the cell-surface class II MHC molecules expressed by the cell lines, **(a)** 1-5.4 ($I\text{-}A^u$) and **(b)** CH27 ($H\text{-}2^k$). Shown is one of five independent experiments, all with equivalent results. Binding levels are expressed as median fluorescence intensity (MFI). Background signal was determined in the absence of biotinylated peptide and has been subtracted from data (1-5.4, 3.40; CH27, 3.05). Cells were incubated with biotinylated peptide for 18 hours at 37°C before flow cytometric analysis (*Chapter 2*, §2.7.2).

allotype (Figures 4.8 & 4.9), *i.e.* poorer binding of the substituted CLIP variants, K83A, K86A and P87A, relative to wild-type CLIP81–104. Similarly, when the same N-terminally-substituted CLIP81–104 analogues were examined for their ability to bind to I-A^k molecules (Figure 4.10b), the substitution of the Lys 86 residue by L-alanine also caused a decline the binding capacity relative to the wild-type sequence. However, this time, the substitution of Lys 83 and Pro 87 led to an increase in the affinity of this sequence for binding to I-A^k. This result was reproduced in five replicate experiments and is consistent with the earlier finding that this allotype exhibited the most marked improvement in binding CLIP when the Pro 87 residue was truncated from the sequence (Tables 4.2–4.4).

The three CLIP residues, Lys 83, Lys 86 and Pro 87, shown herein to modulate the binding of this ligand to I-A molecules, are conserved in mouse, rat, cow and human Ii (*Chapter 1*, Figure 1.5). Of significant note, these three amino acid residues correspond to those identified by Kropshofer *et al.* (1995b) as being able to influence the dissociation rate of human CLIP from HLA-DR molecules. To investigate further the role of these CLIP amino-terminal residues, a second series of N-terminal L-alanine-substituted peptides was synthesised, this time without conjugation to a biotin moiety so as to be suitable for use as competitors in a cell-surface binding assay against the biotinylated wild-type peptide, CLIP81–104. The results of these assays with the cell lines, A20 and CH27, are shown in Figure 4.11. Data are presented as mean percentage inhibition from two independent experiments.

For the A20 cells, the K83A and K86A CLIP analogues both showed a reduced capacity to compete with biotinylated CLIP81–104 relative to the wild-type peptide (Figure 4.11a), consistent with the results obtained with the biotinylated variants (Figure 4.8). However, the Pro 87 substitution this time did not reveal an effect on the binding affinity. The results obtained with the CH27 cell line (Figure 4.11b) were also largely in agreement with previous results (Figure 4.10b). The K83A and P87A substitutions showed an enhanced ability to compete with the biotinylated ligand for binding to the I-A^k allotype, although a modulating effect of the K86A substitution was no longer evident.

Lastly, it was desired to evaluate the ability of the L-alanine-substituted CLIP81–104 analogues to compete for binding to purified I-A class II MHC molecules in an immunoassay at pH 5.0. The results obtained with $\alpha\beta$ dimers of the I-A^d allotype are shown in Figure 4.12, with data presented as mean percentage inhibition calculated from two independent experiments. Under these conditions, no obvious differences were

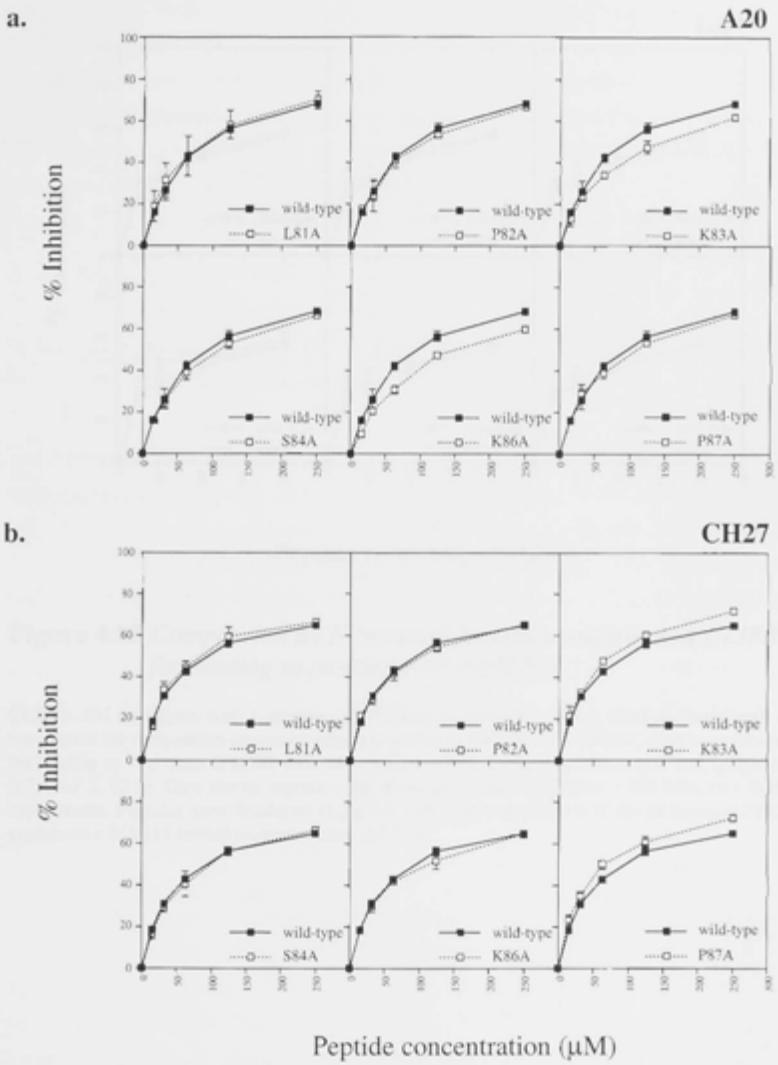


Figure 4.11 *Competition by N-terminal L-alanine-substituted CLIP81–104 for binding to A20 and CH27 cell lines.*

CLIP81–104 analogues with L-alanine substitutions in their N-terminus (dashed lines, open squares) were tested for competition against biotinylated wild-type CLIP81–104 (50 μM; solid line, filled squares) for binding to the cell-surface I-A molecules of the cell lines **(a)** A20 ($H\text{-}2^d$) and **(b)** CH27 ($H\text{-}2^s$). Data shown represent the mean percentage inhibition ± SD from two independent experiments. Cells were incubated with biotinylated peptide and competitor for 18 hours at 37°C before flow cytometric analysis, as described in *Chapter* 2, §2.7.3.

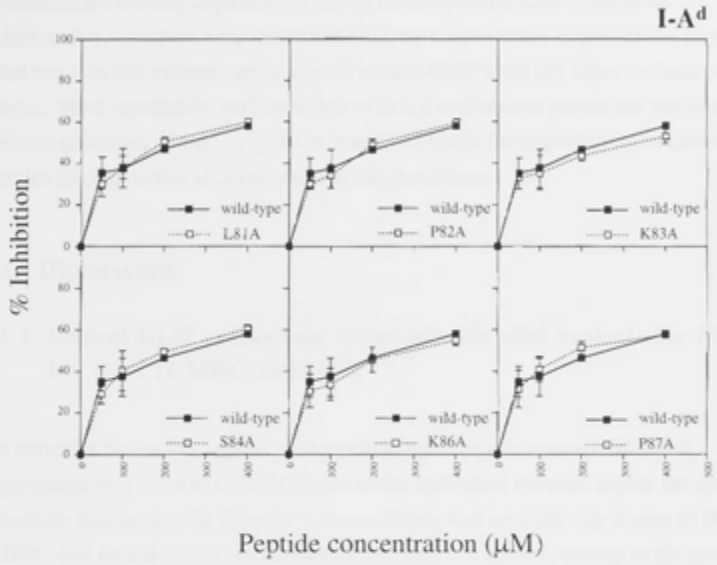


Figure 4.12 *Competition by N-terminal L-alanine-substituted CLIP81–104 for binding to purified I-A^d at pH 5.0.*

CLIP81–104 analogues with L-alanine substitutions in their N-terminus (dashed lines, open squares) were tested for competition against biotinylated wild-type CLIP81–104 (30 μM; solid line, filled squares) for binding to I-A^d class II MHC molecules purified from the BALB/c-derived B cell lymphoma, A20 (*Chapter 2*, §2.6). Data shown represent the mean percentage inhibition ± SD from two independent experiments. Peptides were incubated at pH 5.0 with immunopurified I-A^d for 48 hours at 37°C before capture on a M5/114-coated microtitre plate (§2.7.3).

apparent in the binding capacities of any of the substituted CLIP analogues. Unfortunately, however, time constraints did not allow further experimental assessment of the possible self-release mechanism of mouse CLIP from any other variants of the I-A isotype. Most regrettably, sufficient time was not available to permit the purification of adequate quantities of the I-A^k protein from CH27 cells for a similar examination of these peptides binding to this allotype under acidic conditions.

4.4. Discussion

4.4.1 Central CLIP main-chain atoms provide vital contacts for binding to I-A class II MHC molecules

The attractive forces that uphold an interaction between two amino acid-based biopolymers may involve the side chains of the individual residues and/or the atoms of the polyamide backbones. In *Chapter 3*, it was shown that specific side chains of the CLIP86–104 ligand do not provide significant positive binding energy to the association with mouse I-A class II MHC molecules. This suggests that this peptide must bind to these $\alpha\beta$ dimers predominantly through interactions mediated by its main chain atoms. This possibility has been examined by the experiments described in this chapter using a set of CLIP86–104 analogues exhibiting single D-alanine amino acid substitutions (Table 4.1). Within these peptides, the inverted orientation of the atoms around the chiral α-carbon of the D-alanine residue has been shown to create a conformational disturbance (Figure 4.2) which, in turn, may disrupt the ability of the CLIP main chain atoms to form contacts with residues of the class II MHC heterodimer.

Employing the D-alanine-substituted analogues in competitive binding assays with the molecules, I-A^s, I-A^k and I-A^d, it was found that any configurational substitution within the central region of the CLIP sequence, *i.e.* residues 91–99, reduces the binding potential of this ligand (Figure 4.3–4.6). This is in striking contrast to the results reported in *Chapter 3*, where many side-chain substitutions within this same segment of the CLIP sequence were tolerated (Figures 3.2 & 3.3). Thus, unlike the CLIP side chains within this region, the main-chain atoms do indeed provide a vital contribution to enable this peptide to interact with these class II MHC molecules. Nevertheless, the binding potential of the central sequence element of CLIP alone is not sufficient to sustain the interaction (Table 4.2 & Figure 4.7). Rather, it represents the essential basic framework of the binding motif of this ligand. Experiments with truncated, frameshifted and further substituted CLIP analogues revealed collectively that this CLIP91–99

sequence must be flanked by several additional amino acids in order to bind efficiently to the mouse I-A variants (Tables 4.2–4.4 & Figure 4.7). In particular, 2–3 additional residues at the amino-terminus of this sequence element were required for effective binding, altogether forming a peptide of at least 13 amino acids in length. The inclusion of certain N-terminal residues beyond the main 13-mer binding determinant also appears to modulate the affinity of CLIP for the different I-A molecules (Tables 4.2–4.4 & Figures 4.7–4.11).

The 13-residue minimum length requirement for the CLIP sequence to bind to I-A αβ dimers is consistent with the number of residues deemed necessary to uphold the interaction between human CLIP and the molecules, HLA-DR1, DR2, DR3(17) or DR7 (Geluk *et al.*, 1995; Malcherek *et al.*, 1995). This size also correlates well with that of naturally-processed antigen-derived peptides eluted from the binding groove of different mouse and human class II MHC variants, typically being within 10–28 amino acids in length (Rudensky *et al.*, 1991; Hunt *et al.*, 1992; Chicz *et al.*, 1992, 1993; Rammensee *et al.*, 1995). At the time, these similarities provided yet more support for the notion that the groove was the site of the CLIP interaction. This was soon to be confirmed with the publication of the crystal structure of the CLIP–HLA-DR3 complex (Ghosh *et al.*, 1995).

As described in *Chapter 3* (§3.4.3), X-ray crystallographic analysis revealed that CLIP binds within the peptide-binding groove of the HLA-DR3 αβ dimer in a manner highly similar to that seen previously with the antigenic influenza virus haemagglutinin peptide, HA306–318, complexed with HLA-DR1 (Stern *et al.*, 1994). Firstly, both ligands were noted to have side chains inserted into the polymorphic pockets within this site. Secondly, and of particular interest in this chapter, the main-chain amide protons and carbonyl oxygen atoms of a 12–13 residue stretch of each of the two peptides participated in nearly identical hydrogen-bonding schemes with the residues of the HLA-DR α_1 and β_1 domains. More recently, X-ray crystallography has confirmed that these same two types of interactions also tether peptide ligands within the groove of mouse I-A heterodimers (Fremont *et al.*, 1998b; Scott *et al.*, 1998). Thus, in the context of the results presented herein and previously in *Chapter 3*, it may be interpreted that CLIP binds to the molecules, I-A^u, I-A^k and I-A^d, principally through the formation of an elaborate hydrogen bond network within the peptide-binding groove.

4.4.2 The promiscuous binding of CLIP to I-A class II MHC variants is supported by a sequence-independent network of hydrogen bonds

The dominant contribution of the hydrogen bond network to the association of CLIP with I-A molecules is likely to be a significant factor in the ability of this sequence to bind promiscuously to different class II MHC molecules. This is by virtue of the fact that the formation of such contacts is largely independent of both the sequence of the peptide ligand and the amino acid polymorphism within the groove (Brown *et al.*, 1993). Indeed, of the 17 hydrogen bonds identified in the crystal structure of CLIP bound to HLA-DR3, 11 involved residues that are conserved in most class II MHC molecules, of which 10 took place to main chain atoms of the CLIP backbone (Ghosh *et al.*, 1995). These conserved residues include αAsn 62, αAsn 69, αArg 76 and βAsn 82, all of which are present in the known variants of the I-A allotype (Davis *et al.*, 1989; *Chapter 1*, Figure 1.4).

The capacity of CLIP to form a sufficient number of main chain hydrogen bonds to sustain its interaction with different class II MHC molecules is facilitated by the conformation that it adopts within the peptide-binding groove. Specifically, an analysis of the CLIP sequence complexed with HLA-DR3 reveals that the main chain of the peptide is bound in a straight, extended conformation with a pronounced left-handed twist, such that successive side chains project from the backbone approximately every 128°–130° (Ghosh *et al.*, 1995). This finding is consistent with a prediction from the homology models of Lee & McConnell (1995) that the conformation of this ligand would be the same as that adopted by antigenic peptides binding within this site. In this respect, such an arrangement has been found in all peptide–class II MHC complexes analysed by X-ray crystallography to date (Stern *et al.*, 1994; Ghosh *et al.*, 1995; Fremont *et al.*, 1996, 1998b; Dessen *et al.*, 1997; Scott *et al.*, 1998) and is deemed to be similar to a type II polyproline helix, which characteristically shows an angle of projection of -120° *per* residue (Stern *et al.*, 1994).

The ideal conformation for a ligand to adopt for binding to a class II MHC molecule would be one that provides the constituent amino acid atoms at suitable positions for interacting with the groove residues, while synchronously minimising steric repulsion. That adopted by CLIP and the other peptide ligands would appear to do just this. Taking the first residue in the conformation as that involved in the N-terminal-most hydrogen bond (typically the residue at relative position, P-2; *Chapter 1*, Figure 1.4a), then the periodic rotation of the amino acid side chains of a peptide in the type II polyproline helix

would result in every second or third residue projecting away from the binding site into the solvent (residues P-1, P2, P5, P8, P10; Stern *et al.*, 1994). Meanwhile every third or fourth side chain would be oriented across or downwards into the groove to interact with the polymorphic residues of the MHC pockets (P1, P3/P4, P6, P7, P9). For a bound peptide, this conformation minimises the risk of steric repulsion that may occur if no suitably-accommodating binding pocket is available for the individual side chains by limiting the number of contacts made through the amino acid side-chains. At the same time, the twisting arrangement of peptide backbone causes the outward projection of backbone amide protons and carbonyl oxygen atoms to maximise the opportunity of these atoms to participate in intermolecular hydrogen bonds with the residues of the groove. Consistent with CLIP adopting this conformation, the side chain substitution experiments performed in *Chapter 3* which examine the binding of CLIP to I-A yielded the most notable effects at intervals of every third and/or fourth residue (Figures 3.2, 3.3 & 3.6). A similar result was obtained in equivalent experiments with HLA-DR variants (Geluk *et al.*, 1995; Malcherek *et al.*, 1995). It may be envisaged that an alternative conformation would be less efficient at achieving these aims. For example, an extended β-strand would only permit alternate side chains to project from the groove. Also unfavourable, an α-helix would not enable the peptide backbone to make any bonding interactions with the MHC groove due to the orientation of the main chain atoms along the axis of the helix to form an internal hydrogen bond network (Pauling *et al.*, 1951).

The universal mode in which different ligands appear to bind to class II MHC molecules implies that they all may adopt the common type II polyproline conformation irrespective of the secondary structure of the determinant within the native progenitor protein (Stern *et al.*, 1994; Fremont *et al.*, 1996, 1998b; Dessen *et al.*, 1997; Scott *et al.*, 1998). Regarding the CLIP sequence, nuclear magnetic resonance (NMR) of a soluble recombinant form of the invariant chain protein has shown that the N-terminal third of the lumenal region (residues 58–118) is largely unstructured in solution (Jasanoff *et al.*, 1995, 1999). This is consistent with the finding that this domain is highly susceptibility to proteolytic digestion and potential cleavage sites have been identified for the protease, papain, approximately every ten residues along its length, including a site within CLIP immediately N-terminal to Ala 94 (Park *et al.*, 1995). However, when an interaction is formed with an αβ dimer, the Ii domain encompassing residues, 72–110, becomes ordered rapidly (Jasanoff *et al.*, 1999) and the susceptibility of both free CLIP and Ii to cleavage at this site is lost (Avva & Cresswell, 1994; Park *et al.*, 1995). Resistance to proteolysis has also been reported to be acquired by other peptides when they bind within the class II MHC groove (Donermeyer & Allen, 1989).

The protection of the CLIP Ala 94 papain cleavage site within intact Ii is also seen when Ii trimers are complexed either singly or in trimeric form with HLA-DR3 molecules (Avva & Cresswell, 1994). Together with the overall flexibility of the Ii domain encompassing CLIP (Jasanoff *et al.*, 1995, 1999), this would suggest that the CLIP sequence may bind within the MHC groove very early in the antigen presentation pathway. Indeed, the accessibility of the peptide-binding groove to the CLIP sequence within Ii has been demonstrated by the efficient presentation of antigenic peptides engineered into this protein in place of CLIP (Stumptner & Benaroch, 1997; van Bergen *et al.*, 1997; Barton & Rudensky, 1998; Fujii *et al.*, 1998; Malcherek *et al.*, 1998; ten Bosch *et al.*, 1999). Such modifications may also lead to the formation of αβIi trimers that exhibit stability in the presence of SDS detergent (Stumptner & Benaroch, 1997; Barton & Rudensky, 1998). Nevertheless, experiments performed with a CLIP-specific monoclonal antibody and T cell hybridomas show that, when bound to class II MHC molecules, the CLIP sequence in intact Ii does not assume an identical conformation as the isolated peptide (Morkowski *et al.*, 1995; Eastman *et al.*, 1996). Rather, the cross-reaction of these CLIP-specific reagents with some large Ii–class II MHC proteolytic intermediates such as LIP (Blum & Cresswell, 1988) would indicate that this conformation appears only later in the pathway, once the Ii protein has undergone some degree of proteolytic cleavage. This may be a consequence of the N-terminal residues of the CLIP sequence imposing physical constraints upon the adjacent element within the groove (Stumptner & Benaroch, 1997; see below, §4.4.4).

4.4.3 Secondary structure predictions for Ii show a high degree of accuracy

In this chapter, computational algorithms have been used to provide an estimate of the conformational tendencies of the CLIP sequence (Figure 4.1). From an input file containing the protein primary sequence, such algorithms are able to predict aspects of secondary structure by assessing the given sequence for a range of features. Typically, these take into account such parameters as the fraction of residues of any particular physicochemical type (individual amino acids have different propensities for certain secondary structure states), the position of each residue within the polypeptide chain (residues at the end of chains have greater flexibility due to fewer structural constraints), secondary-structure feedback effects (individual regions of defined secondary structure may influence each other during protein folding) and, also, by comparing the sequence with homologous segments from proteins of known three-dimensional structure. The use

of evolutionary information present in multiple sequence alignments also increases substantially the accuracy levels of the prediction, for example, by providing details of aligned deletions and insertions — these are known to occur more often in loop regions than in internal parts of α-helices and β-strands (Salamov & Solovyev, 1995).

Many automated systems are now available to perform secondary structure prediction algorithms for a protein of choice. The PHDsec method of Rost & Sander (1993, 1994) uses a system of neural networks together with a multiple alignment of the input sequence file from the SWISSPROT sequence database. The NNSSP program similarly employs profiles of multiple sequence alignments, but uses the simpler nearest-neighbour algorithm to score the local structural environment (Salamov & Solovyev, 1995). Both these methods yield similar overall three-state accuracies of prediction (α-helix, β-strand and coil) of Q_3>72%.

The PHDsec and NNSSP methods both predict an equivalent conformational structure for the CLIP sequence whether it is an isolated peptide or encompassed within the intact Ii protein (Figure 4.1). This would suggest that other stretches of significant secondary structure within the intact Ii do not interfere with the ability of the CLIP region to assume this conformation. Although the type II polyproline helix actually adopted by CLIP (Ghosh *et al.*, 1995) is beyond the prediction capabilities of such algorithms — these methods are restricted to giving estimates of regular α-helical or β-sheet domains — this technique nevertheless predicted correctly that this sequence was favoured to assume some form of periodic structure. In this way, secondary structure prediction algorithms provide a useful guideline of conformational tendencies.

As a further indication of the value of these predictions, other aspects of the PHDsec and NNSSP conformational estimates of Ii show good correlation with existing experimental data. For example, protein transmembrane domains typically fold into α-helical segments (Engelman *et al.*, 1986; Singer, 1990) and, indeed, such a conformation is predicted for this region of the Ii protein, residues 32–56 (Figure 4.1). Similarly, the helical stretch that was predicted within the N-terminus of Ii corresponds to the cytoplasmic tail, which contains the two targeting signals that mediate the localisation of this protein in the endocytic compartments, residues Asp 3–Ile 8 and Glu 12–Leu 17 (*Chapter 1*, Figure 1.5; Pieters *et al.*, 1993; Bremnes *et al.*, 1994; Odorizzi *et al.*, 1994; Pond *et al.*, 1995). In agreement with the structural predictions shown in Figure 4.1, the Leu 7–Ile 8 motif has been identified by NMR to lie within a nascent helix, while the membrane proximal motif is part of a turn (Motta *et al.*, 1995). Additionally, it has been reported that a 27-

amino acid peptide corresponding to this region trimerises into an α-helical bundle (Motta *et al.*, 1997).

Both the PHDsec and NNSSP methods also predict a high degree of α-helical content C-terminal to the CLIP sequence (Figure 4.1). The largest stretch of this corresponds well to the exon 6 residues, 163–183, that are involved in the formation of Ii homotrimers (Biljmakers *et al.*, 1994a). Indeed, α-helical content has been detected previously in this region by CD spectroscopy (Park *et al.*, 1995) and NMR (Jasanoff *et al.*, 1998). Moreover, the C-terminal stretch of the lumenal domain, Ii118–193, encompassing the trimerisation signal has been found to be resistant to protease activity, consistent with a compact and well-ordered conformation (Jasanoff *et al.*, 1995; 1999). An earlier analysis of the periodic hydrophobicity in the Ii protein has also revealed the likely presence of an amphipathic helix between residues, 146–164 (Elliott *et al.*, 1987a), later also detected by CD spectroscopy (Lu *et al.*, 1990) and NMR (Jasanoff *et al.*, 1998). This is compatible with the smaller stretch of helicity predicted by both PHDsec and NNSSP between residues, 147–155/156. Similarly, the helical character predicted in Figure 4.1 spanning residues Ii121–132 has been reported previously using NMR techniques (Jasanoff *et al.*, 1998).

4.4.4 A self-release mechanism for CLIP binding to I-A molecules?

In this study, the truncation of the N-terminal residues, Lys 86 and Pro 87, from the mouse CLIP86–104 sequence has been shown to modulate the binding affinity of this ligand for I-A class II MHC molecules (Tables 4.2–4.4 & Figure 4.7). In particular, binding to the allotype, I-A^k, was enhanced when such residues were removed. A similar but less-pronounced effect was also observed for I-A^u molecules but not for I-A^d. By comparison, the removal of the last two C-terminal native residues did not affect the ability of the CLIP sequence to bind to the different I-A class II MHC variants consistently. Further investigations into the role of these residues using single N-terminally-substituted CLIP81–104 analogues confirmed that the Lys 86 and Pro 87 residues did have an influence on the binding of CLIP to I-A^k, this time together with Lys 83 (Figures 4.10 & 4.11). Specifically, the substitution of either Lys 83 and Pro 87 by a single L-alanine residue led to an enhancement in the ability of the CLIP sequence to bind, while a similar substitution of the Lys 86 residue decreased binding. By contrast, for I-A^d and I-A^u molecules, the effect of all these substitutions, if any, was to decrease the ability of these peptides to bind (Figures 4.8–4.12).

The increase in the binding of the CLIP sequence to mouse I-A^k molecules observed when certain native N-terminal residues were deleted (Tables 4.2–4.4 & Figure 4.7) is consistent with the findings of a number of other investigations. For example, Chicz *et al.* (1992) have reported that an equivalently-modified human CLIP ligand, 90–103, binds with higher affinity to HLA-DR1 than the longer, CLIP81–103. Similarly, N-terminally-truncated CLIP ligands have been shown to exhibit better competitive ability for binding to HLA-DR1 and DR7 (Geluk *et al.*, 1995) and to the mouse allotypes, I-A^k, I-A^d and I-A^b (Naujokas *et al.*, 1998) than longer variants. Urban *et al.* (1994) have also reported that human CLIP ligands that exhibit intact N-terminal residues, 81–89, are released preferentially from purified HLA-DR1 at low pH, while those cleaved between Ser 89 and Lys 90 remained bound, *e.g.* 90–103. Lastly, Kropshofer *et al.* (1995a, 1995b) have shown using a cell-free system that the presence of the N-terminal residues in human CLIP increases significantly the dissociation rate of this ligand from an HLA-DR $\alpha\beta$ dimer. For example, the time of half-maximal dissociation, $t_{1/2}$, from soluble, recombinant HLA-DR3 at pH 5.8 was 1.5 hours for the full-length CLIP81–105 ligand, compared with nearly 6 hours for the N-terminally-truncated variant, CLIP90–105 (Kropshofer *et al.*, 1995b).

Of particular interest, the N-terminal CLIP residues reported herein to modulate the binding affinity of mouse CLIP for I-A molecules are the same as those found to enhance the dissociation of human CLIP from HLA-DR, all three being conserved in the Ii sequences of mouse, rat and human (*Chapter 1*, Figure 1.5). Kropshofer *et al.* (1995b) have shown that the introduction of a single L-alanine residue into the human CLIP sequence in place of any of the three residues, Lys 83, Pro 87 or, to a lesser extent, Lys 86, led to an increase in the stability of the CLIP–HLA-DR3 complex similar to that observed with the CLIP90–105 ligand. These authors have proposed that such residues in the human CLIP sequence have a specific role to ensure the efficient release of this ligand independently of the actions of HLA-DM when HLA $\alpha\beta$ dimers encounter antigen-derived ligands in the acidic endosomal compartments. In support of this notion, only 50–70% of the peptides eluted from HLA-DR3 molecules expressed in HLA-DM° cell lines are CLIP variants (Kropshofer *et al.*, 1995b). This would be expected to be nearer to 100% if these heterodimers were entirely reliant upon HLA-DM for facilitating the dissociation of CLIP (Kropshofer *et al.*, 1995a, 1995b).

The results presented in this study (Tables 4.2–4.4 & Figures 4.7–4.11) suggest that a similar mechanism of CLIP self-release may operate when this ligand binds to the mouse class II MHC allotype I-A^k but not in the interaction with I-A^d nor I-A^s. Although

additional experimentation is still needed to substantiate these findings, the possibility that such a mechanism does occur is appealing, particularly when the differences seen in the binding data with the L-alanine N-terminal substitutions between I-A^d and I-A^k are put into context with their different requirements for H-2M or its human equivalent, HLA-DM. The preliminary results on this matter were unclear (Stebbins *et al.*, 1995), however it has been shown more recently that I-A^d molecules expressed in HLA-DM° cell lines exhibit the same phenotype of defective antigen presentation as that described previously for HLA-DR molecules with the same cellular background (Stebbins *et al.*, 1996; Weenink *et al.*, 1997). Specifically, these molecules displayed an inability to form SDS-stable αβ dimers, a failure to present peptides derived from intact antigens, a loss of expression of certain mAb epitopes and were occupied predominantly with CLIP (Mellins *et al.*, 1990, 1991, 1994; Riberdy *et al.*, 1992; Sette *et al.*, 1992a). By contrast, I-A^k molecules expressed in the same HLA-DM° APC showed a much less severe defect in antigen presentation. For example, I-A^k αβ dimers did form SDS-stable dimers, did present at least some determinants derived from protein antigens and were largely free of CLIP (Brooks *et al.*, 1994; Stebbins *et al.*, 1996). Collectively, these findings would suggest that I-A^k molecules differ from those of the I-A^d allotype in that HLA-DM (or H-2M) is not an obligatory requirement for efficient presentation of antigen. In this respect, it is tempting to speculate that the relative allele-specific contributions of the two mechanisms, HLA-DM and CLIP self-release, may be determined by the overall affinity of this ligand for a particular class II MHC heterodimer. For example, the self-release of CLIP may be quite sufficient for class II MHC variants with a low affinity for this ligand, *e.g.* I-A^k, HLA-DR5 (Bangia & Watts, 1995; Liang *et al.*, 1995; Sette *et al.*, 1995; Ramachandra *et al.*, 1996). However, those allotypes to which CLIP binds with higher affinity may still exhibiting some degree of the self-release mechanism but would be more reliant upon HLA-DM to facilitate its dissociation. This would include such molecules as I-A^d, HLA-DR3, DR2 and DR1 (Bangia & Watts, 1995; Kropshofer *et al.*, 1995a, 1995b; Liang *et al.*, 1995; Sette *et al.*, 1995).

Lastly, the substitution of any of the outermost two to three native N-terminal CLIP residues with D-alanine also was shown to increase the ability of CLIP86–104 to compete for antigen presentation (Figures 4.3 & 4.5a) and, to a lesser extent, cell-surface binding (Figures 4.4 & 4.5b). While it is possible that this may again reflect the ability of these native N-terminal residues in CLIP to the reduce the binding affinity of this ligand for mouse I-A molecules this would seem unlikely, since the greatest effect of these substitutions was seen not with I-A^k where this mechanism is predicted to be most efficient, but with the I-A^u and I-A^d allotypes. Most likely, the apparent increase in

binding observed for the D-alanine N-terminal-substituted peptides is merely an artefact of the increased resistance to proteolytic degradation that these residues provide. This theory is supported by the finding that this effect is reduced in the immunoassays conducted with purified MHC proteins where peptidases are less abundant (Figure 4.6). The strategy of introducing D-amino acids into peptide termini to protect them from proteolysis has been used to great effect in creating ligands that exhibit increased stability in serum-containing medium (Lamont *et al.*, 1990; Powell *et al.*, 1993; Alexander *et al.*, 1994) and may indeed improve their inhibitory abilities. The role of the protective D-alanine species in these CLIP ligands in the experiments described here may be confirmed by conducting a binding analysis in the presence of protease inhibitors or using fixed APC in serum-free medium.

4.5. Conclusions

The experiments described in this chapter have elucidated a binding motif for the association of mouse CLIP with I-A class II MHC molecules, in particular, a critical role for the peptide backbone conformation has been established. Using a set of configurationally-substituted CLIP86–104 analogues, together with different truncated variants, in a series of competitive binding assays it was apparent that CLIP interacts with the three molecules, I-A^d, I-A^s and I-A^k, by a similar motif. Binding occurs through a continuous 13-residue sequence element encompassing the highly phylogenetically-conserved residues, 91–99. Consistent with this, the X-ray crystallographic analysis of human CLIP complexed with the human class II MHC molecule, HLA-DR3, identifies this same stretch of residues as occupying the peptide-binding groove (Ghosh *et al.*, 1995). Extrapolating from this crystal structure, the sensitivity to configurational substitutions of these residues in mouse CLIP lies in the participation of their main-chain atoms in hydrogen bonds with conserved residues of the I-A binding groove, in an array which has an absolute requirement for a specific conformation.

Additional to the interactions of CLIP within the groove, an examination of the binding of length-altered and/or side-chain-substituted analogues suggests that N-terminal CLIP residues that contact the $\alpha\beta$ dimer outside of this site may also modulate the affinity of this peptide. These early results appear to be consistent with the ability of CLIP to facilitate its own release from HLA-DR molecules (Kropshofer *et al.*, 1995a; Kropshofer *et al.*, 1995b) but more study is needed to confirm these findings for the variants of the I-A isotype.

The conserved main-chain hydrogen-bonding system, together with the lack of significant side-chain anchor interactions between CLIP and I-A $\alpha\beta$ dimers (*Chapter 3*) enables this ligand to bind promiscuously to many different I-A allelic variants in a conformation that constitutes a universal binding determinant. It was therefore of interest to determine whether CLIP uses a similar mode of binding in its interaction with mouse class II MHC molecules of the I-E isotype. This possibility is examined in *Chapter 5*.

CHAPTER 5.

The binding of CLIP to mouse I-E class II MHC molecules

5.1. Introduction

Sequence polymorphism in the α_1 and β_1 domains of class II MHC molecules is significant in that it enables the different allelic variants expressed by an individual to present distinct subsets of antigen-derived peptides to the $CD4^+$ T lymphocyte population (Hurley & Steiner, 1995). However, mouse class II MHC molecules of the I-E isotype differ from their I-A counterparts in that their α-chain sequence is largely invariant (reviewed by Mengle-Gaw & McDevitt, 1985). Indeed, of the Eα alleles that have been sequenced, only Eα^a shows any degree of variation within the first domain of the polypeptide, differing by just two amino acids (Ayane *et al.*, 1986; for a description of the domain organisation of the class II MHC $\alpha\beta$ dimer, refer to *Chapter 1*, Figure 1.3). This would be expected to reduce considerably the extent of heterogeneity within the peptide-binding groove between different I-E allotypes. At the time of commencing this study, little was known of the structure of the I-E heterodimer or its mode of binding peptides. It was therefore of interest to determine whether I-E molecules bind CLIP in the same manner as that elucidated for allelic variants of the I-A isotype in *Chapters 3 & 4*.

Previous experimental findings suggested that CLIP may not bind well to I-E class II MHC $\alpha\beta$ dimers. Firstly, the elution of naturally-processed peptides from these molecules fails to yield any peptides nested around the CLIP sequence, such as those found with HLA-DR, DQ and I-A (Rudensky *et al.*, 1991; Chicz *et al.*, 1992, 1993, 1994; Hunt *et al.*, 1992; Riberdy *et al.*, 1992; Sette *et al.*, 1992a; Vartdal *et al.*, 1996). Secondly, synthetic CLIP ligands equivalent to those eluted from I-A variants display only a low affinity for purified I-E molecules but bind well to I-A under the same experimental conditions (Hunt *et al.*, 1992). Lastly, it was noted in *Chapters 3 & 4* of this study that there appeared to be very little, if any, evidence of CLIP binding to the cell-surface I-E molecules expressed by the $I\text{-}A^+/I\text{-}E^+$ cell lines, CH27 ($H\text{-}2^k$) and A20 ($H\text{-}2^d$) in flow cytometry-based assays, whereas interactions with the I-A heterodimers were detected readily by this approach. The general incapacity of CLIP to associate with I-E MHC molecules under these experimental conditions is somewhat unexpected given the ability to co-immunoprecipitate the intact Ii protein with molecules of this isotype in the presence of 0.5% NP-40 detergent (Jones *et al.*, 1978a). A thorough examination of the binding interaction of CLIP with I-E molecules was needed to explain these observations.

It is interesting to note that a number of inbred mouse strains have been described that do not express cell-surface I-E class II MHC molecules, *i.e.* the E^o or E-null phenotype (Jones *et al.*, 1978b, 1981; Mathis *et al.*, 1983). This defect is also estimated to affect up

to 20% of wild mouse populations (Klein & Figueroa, 1981) and has also been reported in other rodent species (Nizetic *et al.*, 1987). It is most often related to mutations or deletions in the genetic locus that encodes the Eα polypeptide (Mathis *et al.*, 1983; Klein, 1986). The reverse situation has not been found, *i.e.* the natural occurrence of I-A° mice. In terms of how the lack of I-E expression may affect the adaptive immune response within these mice, Cosgrove *et al.* (1992) have reported that I-E and I-A αβ dimers are mostly functionally equivalent, at least in terms of correcting the irregularities in the immune system of class II MHC-deficient mice. However, the expression of I-E transgenes in the E° non-obese diabetic (NOD) mouse has been shown to prevent the development of overt diabetes (Nishimoto *et al.*, 1987). Similarly, other mouse strains that lack functional I-E molecules have been reported to be more prone to developing spontaneous autoreactivity (Li *et al.*, 1993). Given the central role of Ii and CLIP in the class II MHC antigen presentation pathway, a detailed examination of the mode of CLIP binding to I-E molecules for comparison with that of I-A may help to illuminate how these molecules might differ both in their respective roles during antigen presentation and, potentially, in the development of autoimmunity.

The experiments described in this chapter have sought to analyse the association of CLIP with allelic variants of mouse class II MHC isotype, I-E. The initial task of this investigation was to examine this interaction by three different experimental approaches in an effort to find an effective means of measuring the binding of CLIP to I-E molecules. Based on the findings of this analysis, the mode of interaction between CLIP and I-E has been defined using both substituted and/or truncated CLIP analogues in immunoassays with purified I-E^d proteins at pH 5.0. The details of this binding motif are compared with those obtained in *Chapters 3* & *4* for the binding of CLIP to mouse I-A molecules. A possible reason for the inability to detect the binding of CLIP to I-E in previous assay methods is discussed.

5.2. Materials and Methods

All experimental procedures performed in this chapter are described in detail in *Chapter 2*. Briefly, the mouse B cell lines A20 (H-2^d) and CH27 (H-2^k) were used as APC in antigen-presentation assays, together with M12.A3 (I-E^d) in cell-surface peptide binding assays (Table 2.1). The T cell hybridomas, 13.26 (I-E^d-restricted, SWM132–147-specific), λ12–26 (I-E^d-restricted, λrep12–26-specific), 2B4 (I-E^k-restricted, mCytc88–103-specific), 4C1.6 (I-E^k-restricted, HEL105–120-specific), 3DO-54.8 (I-A^d-restricted, Ova323–339-specific) and 3A9 (I-A^k-restricted, HEL46–61-specific) were used in the

antigen-presentation assays (Table 2.2), together with the IL-2-dependent T cell line, HT-2. Mouse I-E^d molecules were purified from detergent-solubilised membrane preparations from the BALB/c-derived B cell lymphoma, A20, by immunoaffinity chromatography on a 14.4.4S mAb column (§2.6). I thank Mrs Y. M. Gautam (Laboratory technician) for purifying these proteins.

The L-alanine and D-alanine-substituted CLIP86–104 analogues have been listed previously in *Chapter 3*, Table 3.1 and *Chapter 4*, Table 4.1, respectively. The truncated and frameshifted CLIP analogues are listed in Tables 5.1 and 5.2, respectively, with additional substituted and/or length-altered CLIP ligands detailed in Table 5.3. CLIP81–104 analogues exhibiting single amino-terminal L-alanine substitutions are shown in *Chapter 4*, Table 4.5. Peptide concentrations used in the binding assays are stated in the figure legends. Briefly, competitor peptides were tested over the concentration range 6–400 μM in antigen-presentation assays (§2.7.1) and between 25–400 μM for cell-surface binding assays (§2.7.2) and immunoassays with purified class II MHC molecules (§2.7.1). I wish to extend my thanks to Mrs Y. M. Gautam for performing the experiments shown in Figures 5.5 & 5.6, which illustrate the relative binding affinity of different peptides to purified I-E^d molecules as a function of pH.

5.3. Results

5.3.1 CLIP competes poorly for binding to I-E molecules in both antigen-presentation and cell-surface binding assays at pH 7.0

Given the promiscuous nature of the binding of CLIP to different class II MHC molecules, it may be expected that this ligand would associate with all the different types of αβ dimers present at the surface of cells in a flow cytometry-based peptide binding assay. Nevertheless, when biotinylated CLIP was incubated with A20 cells (I-A^d/I-E^d) in *Chapters 3* (§3.3.1) & *4* (§4.3.1), the level of fluorescence that was generated suggested that this ligand was not binding to the I-E molecules, nor contributing to the pattern of competition by the L- or D-alanine-substituted CLIP analogues. To investigate these findings, CH27 and A20 cells were incubated with biotinylated CLIP86–104 and one of a range of different I-E- or I-A-binding peptides (Figure 5.1). The ability of the I-A-binding peptides to inhibit the biotinylated CLIP signal *versus* those known to bind to I-E should reflect the relative contributions of CLIP associating with each of these isotypes.

For the CH27 cell line (Figure 5.1a), the HEL46–61 peptide (open triangles) effectively

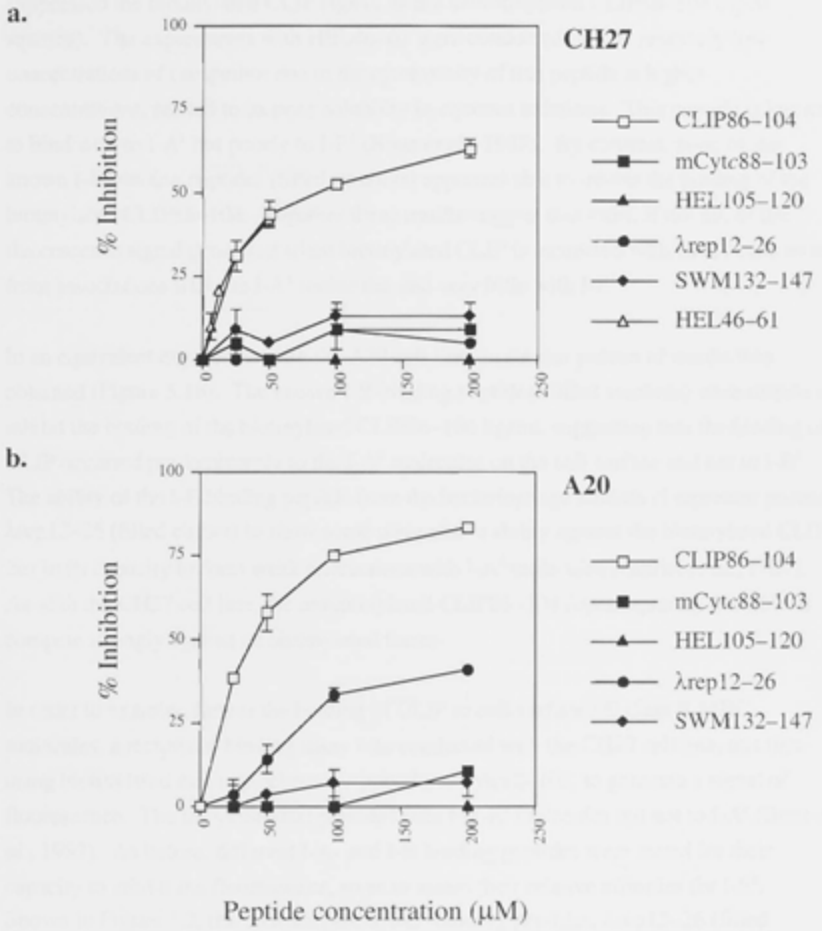


Figure 5.1 *Competition against biotinylated CLIP86–104 for binding to cell-surface mouse class II MHC molecules at pH 7.0.*

The ability of CLIP86–104 to inhibit the cell-surface binding of biotinylated CLIP86–104 (50 μM) to **(a)** CH27 and **(b)** A20 cells was examined and compared with a panel of antigen-derived peptides, mCytc88–103 ($I\text{-}E^{k}$), HEL105–120 ($I\text{-}E^{d,k}$), λrep12–26 ($I\text{-}E^{d,k}$, $I\text{-}A^{d}$), SWM132–147 ($I\text{-}E^{d}$), Ova323–339Y ($I\text{-}A^{d,k}$) and HEL46–61 ($I\text{-}A^{d,k}$). Data shown represent the mean percentage inhibition ± SD from two independent experiments. Cells were incubated with biotinylated peptide and competitor for 18 hours at 37°C before flow cytomteric analysis, as described in *Chapter 2*, §2.7.2.

suppressed the biotinylated CLIP signal, as did unbiotinylated CLIP86–104 (open squares). The experiments with HEL46–61 were conducted only at relatively low concentrations of competitor due to the cytotoxicity of this peptide at higher concentrations, related to its poor solubility in aqueous solutions. This peptide is known to bind well to I-A^k but poorly to I-E^k (Buus *et al.*, 1987). By contrast, none of the known I-E-binding peptides (filled symbols) appeared able to inhibit the binding of the biotinylated CLIP86–104. Together these results suggest that most, if not all, of the fluorescence signal generated when biotinylated CLIP is incubated with CH27 cells arises from associations with the I-A^k molecules and very little with I-E^k.

In an equivalent experiment with the A20 cell line, an similar pattern of results was obtained (Figure 5.1b). The known I-E-binding peptides (filled symbols) were unable to inhibit the binding of the biotinylated CLIP86–104 ligand, suggesting that the binding of CLIP occurred predominantly to the I-A^d molecules on the cell surface and not to I-E^d. The ability of the I-E-binding peptide from the bacteriophage lambda *c*I repressor protein, λrep12–26 (filled circles) to show some competitive ability against the biotinylated CLIP lies in its capacity to form weak interactions with I-A^d molecules (Guillet *et al.*, 1987). As with the CH27 cell line, the unbiotinylated CLIP86–104 (open squares) was able to compete strongly against its biotinylated form.

In order to examine further the binding of CLIP to cell-surface I-E class II MHC molecules, a reciprocal binding assay was conducted with the CH27 cell line, this time using biotinylated moth cytochrome *c* peptide, mCyt*c*88–103, to generate a signal of fluorescence. The mCyt*c*88–103 peptide binds to I-E^k molecules but not to I-A^k (Buus *et al.*, 1987). As before, different I-A- and I-E-binding peptides were tested for their capacity to inhibit the fluorescence, so as to assess their relative affinities for I-E^k. Shown in Figure 5.2, the unbiotinylated, I-E^k-binding peptides, λrep12–26 (filled triangles) and mCyt*c*88–103 (filled squares) competed well with the biotinylated ligand. As expected, the I-A-binding peptides, HEL46–61 (filled circles) and Ova323–339Y (filled diamonds) did not inhibit significantly the biotinylated mCyt*c*88–103 signal. Unbiotinylated CLIP86–104 (open squares) was also a relatively poor competitor for binding to I-E^k, yielding just 44 ± 7.5% inhibition at 400 μM compared with both the λrep12–26 and mCyt*c*88–103 peptides which exhibited 50% inhibitory capacity of the biotin signal at approximately 75 μM and 140 μM, respectively. This result establishes that CLIP is capable of forming complexes with cell-surface I-E class II MHC molecules but that this interaction is of a low affinity. In this respect, the signal derived from the binding of CLIP to I-E molecules in Figure 5.1 is likely to have been overshadowed by that from the higher affinity interactions of this ligand with the I-A heterodimers.

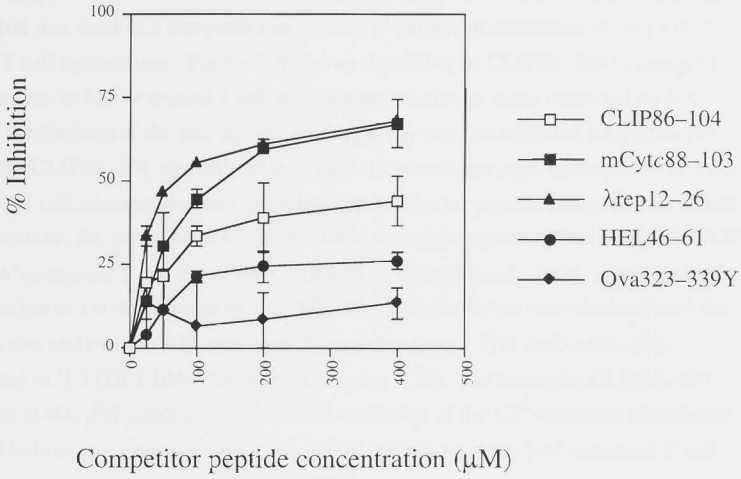


Figure 5.2 *Competition against biotinylated mCytc88–103 for binding to cell-surface I-E^k molecules at pH 7.0.*

The ability of CLIP86–104 to inhibit the binding of biotinylated mCytc88–103 (25 μM) to class II MHC molecules on the surface of CH27 cells was examined and compared in a competition assay with a panel of antigen-derived peptides, mCytc88–103 (I-E^k), λrep12–26 (I-E^k), HEL46–61 (I-A^k) and Ova323–339Y (I-A^k). Data shown represent the mean percentage inhibition ± SD from two independent experiments. CH27 cells were incubated with biotinylated peptide and competitor for 18 hours at 37°C before flow cytometric analysis, as described in *Chapter* 2, §2.7.2.

As an alternative means to investigate the binding of CLIP to cell-surface I-E molecules, CLIP86–104 was used as a competitor in a series of antigen-presentation assays with I-E-restricted T cell hybridomas. Figure 5.3a shows the ability of CLIP86–104 to compete for presentation to I-E^d-restricted T cell hybridomas relative to those restricted by I-A^d. Similar to the findings of the cell-surface binding assay with biotinylated mCytc88–103 (Figure 5.2), CLIP86–104 was only able to inhibit a maximum approaching 50% of the I-E-restricted T cell stimulation, even at the highest competitor peptide concentration of 400 μM. By contrast, the presence of CLIP86–104 in an antigen-presentation assay with A20 and the I-A^d-restricted T cell hybridoma, 3DO-54.8, yielded nearly 100% inhibition of T cell stimulation at a concentration of just 100 μM. A similar result was obtained with the CH27 cell line and the two I-E^k-restricted T cell hybridomas, 2B4 (mCytc88–103-specific) and 4C1.7 (HEL105–120-specific; Figure 5.3b). For example, CLIP86–104 competition at 400 μM yielded just 33 ± 7.6% inhibition of the I-E^k-restricted stimulation of the 2B4 hybridoma, compared with 94 ± 1.7% inhibition of the I-A^k-restricted T cell stimulation with the same concentration of CLIP.

Finally, the binding of CLIP to I-E αβ dimers was examined using a recently-acquired cell line that expresses only class II MHC molecules of the I-E isotype at the cell surface. This cell line, M12.A3, is derived from the H-2^d B cell lymphoma, M12.4.1, but exhibits a defect in the gene encoding the $A\beta^d$ polypeptide (Glimcher *et al.*, 1985). Accordingly, these cells stain positive with the I-E-specific mAb, 14.4.4S, and negative for the I-A^d-specific mAb, MK-D6 (Figure 5.4a). A cell-surface binding assay with M12.A3 cells confirmed that biotinylated CLIP binds very poorly to the cell-surface I-E^d molecules under the conditions of this assay (Figure 5.4b). Data is shown in comparison to an equivalent experiment with the A20 cell line.

5.3.2 CLIP binds well to purified I-E^d molecules at pH 5.0

The findings of the antigen-presentation and cell-surface peptide binding assays suggested that CLIP had a low affinity for mouse I-E MHC molecules, at least under the conditions of these two experimental approaches (Figures 5.1–5.4). In this respect, both assay methods examine peptide–MHC interactions in a cellular context within growth medium buffered at neutral pH (*Chapter 3*, §3.4.1). However, it has been observed frequently that the formation of peptide–class II MHC complexes may be enhanced markedly at low pH, equivalent to the conditions found within the endocytic compartments in which peptides are loaded *in vivo* (*e.g.* Jensen, 1990, 1991; Harding *et al.*, 1991; Wettstein *et al.*, 1991; Mouritsen *et al.*, 1992; Sette *et al.*, 1992b). It was therefore of interest to

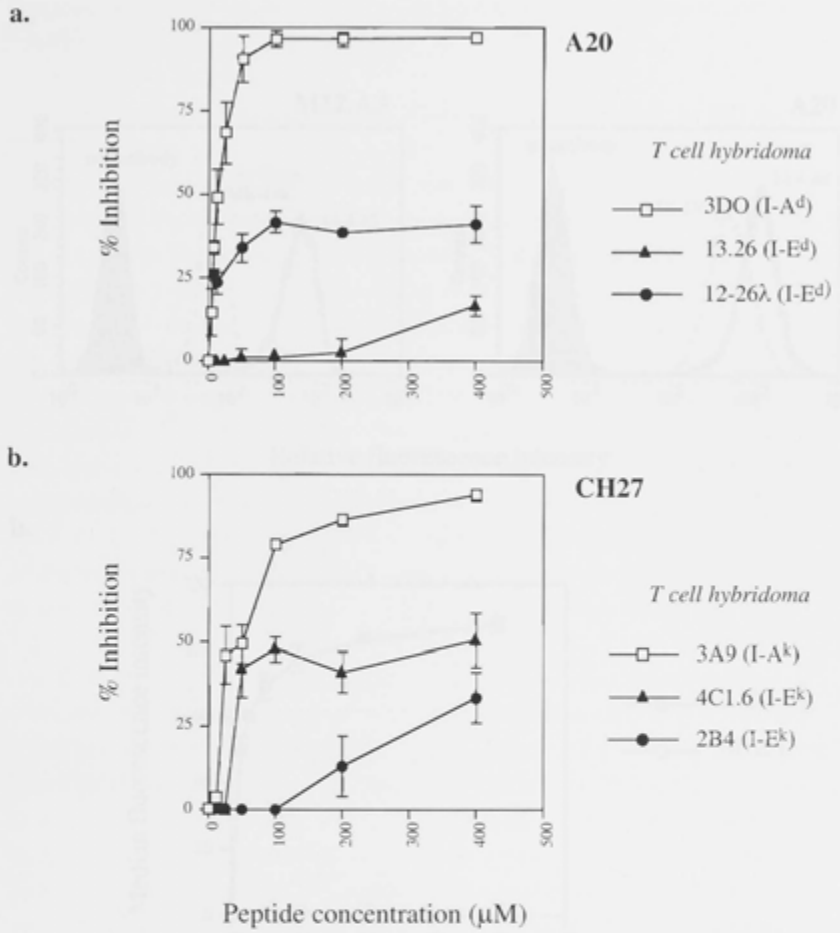
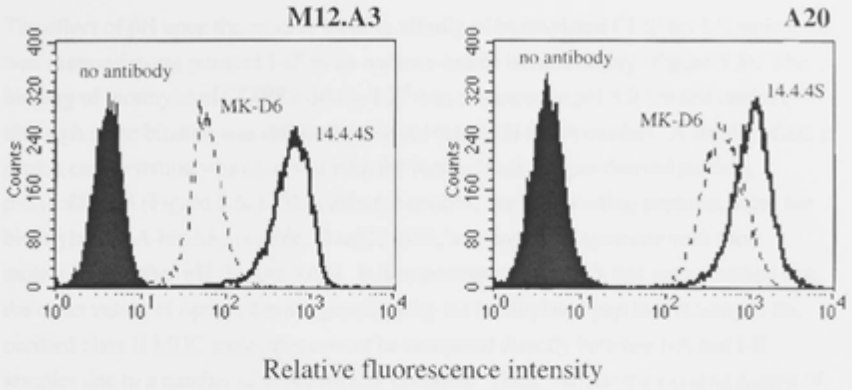


Figure 5.3 *Competition by CLIP86–104 against the presentation of antigen to I-E-restricted T cell hybridomas at pH 7.0.*

CLIP86–104 was tested as a competitor for presentation by the cell lines **(a)** A20 and **(b)** CH27, to the I-E-restricted T cell hybridomas, 13.26 (I-E^d-restricted, SWM132–147, 1.25 μg/mL), 12-26λ (I-E^d-restricted, λrep12–26, 0.05 μM), 4C1.6 (I-E^k-restricted, HEL105–120, 0.025 μM) and 2B4 (I-E^k-restricted, mCytc88–103, 0.3 μM). The competitive ability of CLIP86–104 against I-A-restricted antigen presentation is shown for each cell line for comparison (3DO-54.8: I-A^d-restricted, Ova323–339Y, 0.05 μM; 3A9: I-A^k-restricted, HEL46–61, 1.5 μM). Data are presented as the mean percentage inhibition ± SD from two independent experiments. Cells were incubated with antigen-derived peptide and CLIP competitor for 18 hours at 37°C before the supernatant was harvested and assayed for IL-2, as described in *Chapter 2*, §2.7.1.

a.



b.

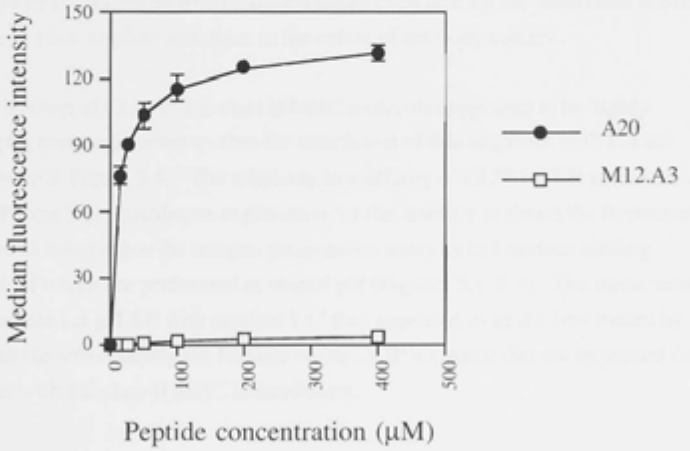


Figure 5.4 *The binding of biotinylated CLIP86–104 to cell-surface I-E^d class II MHC molecules at pH 7.0.*

(**a**) Cells of the H-2^d B lymphoblastoid line, M12.A3, express surface I-E^d molecules, as shown by staining with the I-E-specific mAb, 14.4.4S. However, few I-A^d molecules are detected with the mAb, MK-D6, as a result of a defect in the gene encoding the β-chain of the I-A heterodimer (Glimcher *et al.*, 1985). By contrast, A20 cells express both I-E^d and I-A^d. (**b**) The binding of biotinylated CLIP86–104 was examined to the cell-surface class II MHC molecules of the M12.A3 and A20 cell lines. Shown is one of two independent experiments, both yielding equivalent results. Cells were incubated with biotinylated peptide for 18 hours at 37°C before flow cytometric analysis, as described in *Chapter 2*, §2.7.2.

determine whether the binding of CLIP to I-E molecules would be facilitated under acidic conditions.

The effect of pH upon the relative binding affinity of biotinylated CLIP for I-E molecules was assessed using purified I-E^d in an enzyme-linked immunoassay (Figure 5.5). The binding of biotinylated CLIP86–104 to I-E^d was enhanced at pH 5.0 (closed circles), although some binding was still detectable at neutral pH (open circles). A similar effect of proton concentration was observed with the biotinylated antigen-derived peptide, mCytc88–103 (Figure 5.6a). This effect is specific for I-E^d-binding peptides, since the biotinylated I-A-binding peptide, Ova322–339, was unable to associate with these molecules at either pH (Figure 5.6b). It is important to note with this assay method that the exact values of optical density generated by the biotinylated peptides binding to the purified class II MHC molecules cannot be compared directly between I-A and I-E samples due to a number of experimental variables. These include the existing degree of peptide occupancy of the purified heterodimers and the specific affinity of the capturing mAb, M5/114, for the different molecules. Similarly, optical density measurements cannot always be compared between different plates even bearing the same class II MHC allotype, due to plate-to-plate variations in the extent of antibody coating.

Overall, the binding of CLIP to I-E class II MHC molecules appeared to be highly sensitive to pH, seemingly more so than the interaction of this sequence with I-A $\alpha\beta$ dimers (*Chapter 3*, Figure 3.4). The relatively low affinity of CLIP for I-E molecules at neutral pH (Figure 5.5) provides an explanation for the inability to detect the formation of these complexes using either the antigen-presentation assay or cell-surface binding method, both of which are performed at neutral pH (Figures 5.1–5.4). The immunoassay protocol conducted at pH 5.0 with purified I-E^d thus appeared to be the best means by which to examine subsequently the features of the CLIP sequence that are important for its interaction with I-E class II MHC heterodimers.

5.3.3 A binding motif for the interaction of CLIP with I-E^d at pH 5.0

In order to define the features of the CLIP sequence necessary for binding to mouse I-E MHC molecules, the same series of substituted and/or truncated CLIP analogues were used, as described previously with I-A variants in *Chapters 3 & 4*. Firstly, the D-alanine-substituted CLIP86–104 analogues shown in *Chapter 4*, Table 4.1 were used to examine the conformation-dependent role of the peptide backbone in the interaction with purified I-E^d in an immunoassay at pH 5.0. Shown in Figure 5.7 are the results of one such assay,

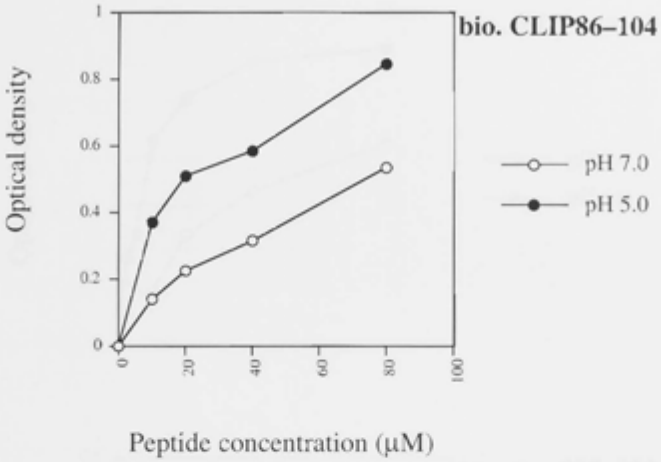


Figure 5.5 *The binding of CLIP86–104 to purified I-E^d.*

Biotinylated mouse CLIP86–104 was incubated with mouse I-E^d class II MHC molecules at 37°C for 48 hours at pH 7.0 (open circles) or pH 5.0 (closed circles) in an ELISA-type assay, as described in *Chapter 2*, §2.7.3. I-E^d molecules were affinity-purified from the BALB/c-derived B cell lymphoma, A20. Shown is one of two independent experiments with equivalent results. Binding is expressed in optical density (OD) units, read at 405 nm with a reference wavelength of 495 nm. The signal from non-specific associations was determined from the binding of biotinylated peptide in the absence of MHC molecules and has been subtracted from data shown (pH 7.0, 0.073 OD units; pH 5.0, 0.066 OD units).

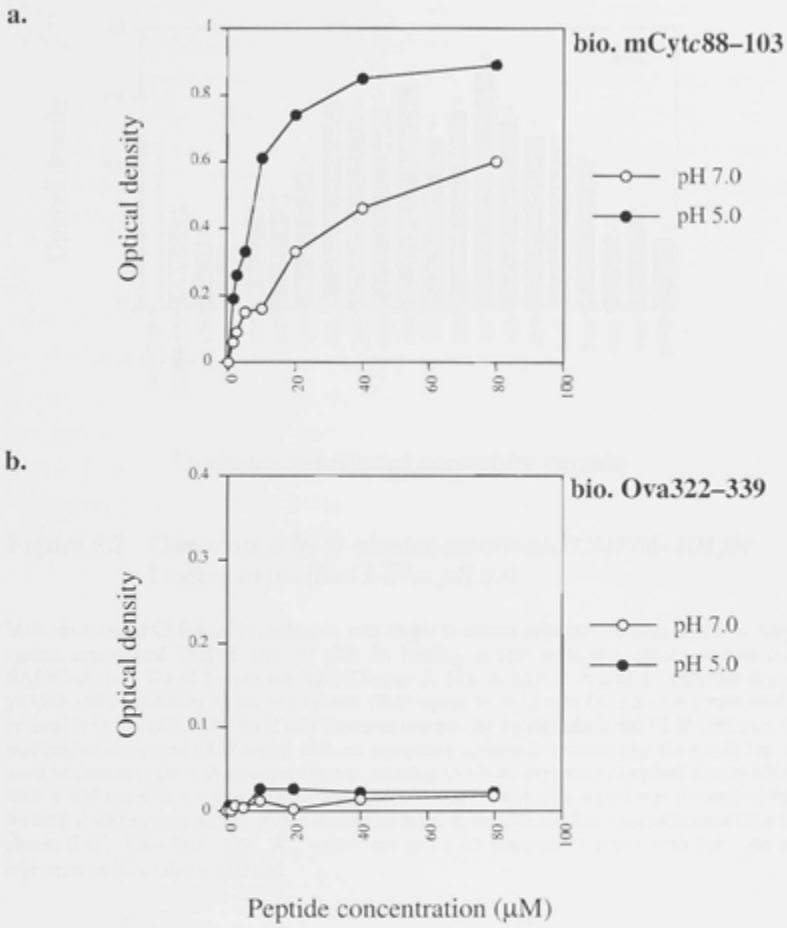


Figure 5.6 *The binding of antigen-derived peptides to purified I-E^d.*

Biotinylated peptides **(a)** mCytc88–103 and **(b)** Ova322–339 were incubated with I-E^d class II MHC molecules at 37°C for 48 hours at pH 7.0 (open circles) or pH 5.0 (closed circles) in an ELISA-type assay, as described in *Chapter 2*, §2.7.3. I-E^d proteins were affinity-purified from the BALB/c-derived B cell lymphoma, A20. Shown for each peptide is one of two independent experiments with equivalent results. Binding is expressed in optical density (OD) units, read at 405 nm with a reference wavelength of 495 nm. The signal from non-specific associations was determined from the binding of biotinylated peptide in the absence of MHC molecules and has been subtracted from data shown (pH 7.0, 0.073 OD units; pH 5.0, 0.066 OD units).

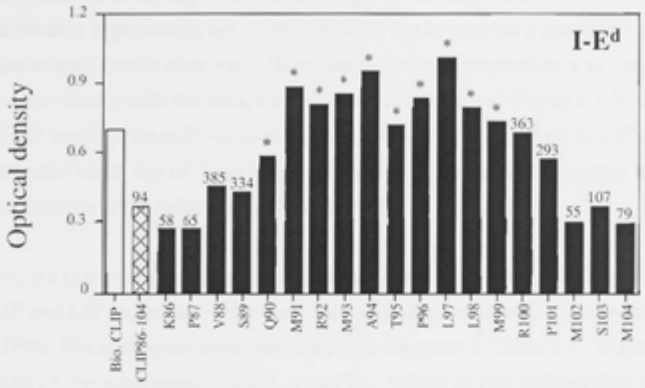


Figure 5.7 *Competition by D-alanine-substituted CLIP86–104 for binding to purified I-E^d at pH 5.0.*

Multiple doses of CLIP86–104 analogues with single D-alanine substitutions were tested as competitors against biotinylated CLIP86–104 (30 μM) for binding to I-E^d molecules affinity-purified from the BALB/c-derived B cell lymphoma, A20 (*Chapter 2*, §2.6 & §2.7.3). A single competitor dose which yielded ~50% inhibition of the biotinylated CLIP signal by wild-type CLIP86–104 (cross-hatched fill pattern) is shown (150 μM). Solid bars represent competition by the substituted CLIP analogues and the maximum biotinylated CLIP signal with no competitor present is indicated by the empty bar. Assays were performed twice with consistent results. Binding levels are expressed as optical density (OD) units, read at 405 nm with a reference wavelength of 495 nm. Non-specific signal was determined from the binding of biotinylated peptide in the absence of MHC molecules and has been subtracted from the data shown (I-E^d, 0.066 OD units). IC_{50} values are given for competitors above each bar. An asterisk represents an IC_{50} value >1000 μM.

representative of the experimental findings. Mean IC_{50} values, calculated from two independent experiments are given atop each bar to provide a quantitative representation of the overall trends observed. Here, the pattern of competition was consistent with that seen previously with the three I-A allotypes in *Chapter 4* (Figures 4.3–4.6). The ability of these configurationally-altered ligands to compete for binding to I-E^d molecules was eliminated when any of these single substitutions fell within the central region of the peptide, between residues Gln 90 and Met 99.

Next, the contributions of the individual peptide side chains to the interaction between CLIP and I-E^d molecules were examined using the series of L-alanine-substituted CLIP86–104 analogues listed previously in *Chapter 3*, Table 3.1. Figure 5.8 shows the results of one such assay. Again, mean IC_{50} values of two independent experiments are provided atop each bar. Most notably, the substitution of several of the individual wild-type CLIP amino acid residues, Met 91, Thr 95, Leu 98 and Met 102 eliminated completely the ability of these particular analogues to compete for binding. The substitution of the Met 99 residue by L-alanine also led to a decrease in the capacity of the CLIP sequence to bind to the I-E^d molecules. By contrast, substitution of Pro 96 resulted in an improvement in binding.

Lastly, the minimum length of the CLIP sequence required to sustain efficient binding to I-E^d molecules was assessed using the set of truncated peptides listed in Table 5.1. The data for the purified I-E^d at pH 5.0 are presented as mean IC_{50} values ± standard deviation from two independent experiments and are shown in comparison to those obtained with purified I-A^d under the same conditions (discussed previously in *Chapter 4*, §4.3.2). As found for the I-A^d molecules, the CLIP sequence was unable to bind to I-E^d when it was truncated to 13 residues or below, *i.e.* peptides, CLIP89–101, 90–100 and 91–99. However, further interpretation of these results is limited by the large experimental errors observed in these experiments. For example, it is not possible to determine the exact effects of removing the outermost residues of the CLIP sequence upon the binding to the I-E^d molecules, as done previously for variants of the I-A isotype in *Chapter 4* (§4.3.2).

5.3.4 Conserved N-terminal residues modulate binding to I-E^d

As discussed in *Chapter 4* (§4.4.4), Kropshofer *et al.* (1995a, 1995b) have proposed that the N-terminal residues of human CLIP may provide a self-release mechanism to enable this ligand to bring about its dissociation from HLA-DR class II MHC molecules independently of the actions of HLA-DM. Experiments in that chapter addressed the

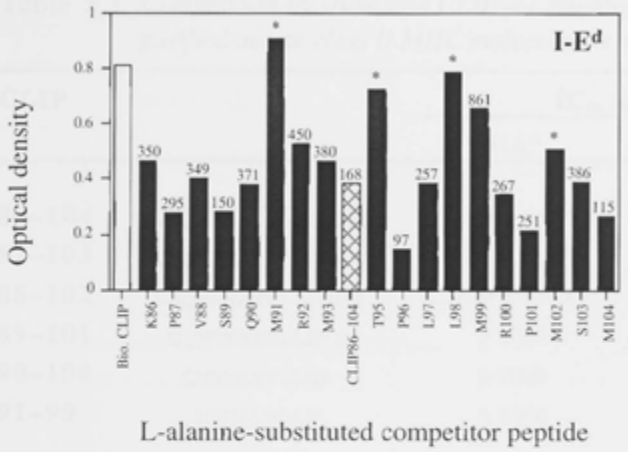


Figure 5.8 *Competition by L-alanine-substituted CLIP86–104 for binding to purified I-E^d at pH 5.0.*

Multiple doses of CLIP86–104 analogues with single L-alanine substitutions were tested for competition against biotinylated CLIP86–104 (30 μM) for binding to I-E^d affinity-purified from the BALB/c-derived B cell lymphoma, A20 (*Chapter 2*, §2.6 & 2.7.3). A single competitor dose which yielded ~50% inhibition of the biotinylated CLIP signal by wild-type CLIP86–104 (cross-hatched fill pattern) is shown (300 μM). Solid bars represent competition by the substituted CLIP analogues and the maximum biotinylated CLIP signal with no competitor present is indicated with the empty bar. Binding assays were performed twice with consistent results. Binding levels are expressed as optical density (OD) units, read at 405 nm with a reference wavelength of 495 nm. Non-specific signal was determined from the binding of biotinylated peptide in the absence of MHC molecules and has been subtracted from the data shown (I-E^d, 0.088 OD units). IC_{50} values are given for competitors above each bar. An asterisk represents an IC_{50} value >1000 μM.

Table 5.1 *Competition by truncated CLIP for binding to affinity-purified mouse class II MHC molecules at pH 5.0.*

CLIP		IC_{50} (μM)	
		I-A^d	I-E^d
86–104	KPVSQMRMATPLLMRPMSM	91.19 ± 64.12	272.50 ± 67.15
87–103	PVSQMRMATPLLMRPMS	310.30 ± 369.90	248.50 ± 177.00
88–102	VSQMRMATPLLMRPM	224.70 ± 150.15	212.90 ± 162.65
89–101	SQMRMATPLLMRP	> 1000	> 1000
90–100	QMRMATPLLMR	> 1000	> 1000
91–99	MRMATPLLM	> 1000	> 1000

Residues were sequentially removed pairwise from each end of the CLIP86–104 sequence. The truncated CLIP were then tested at multiple doses as competitors against biotinylated CLIP86–104 (30 μM) for binding to the affinity-purified murine class II MHC molecules, I-A^d and I-E^d, in an immunoassay at pH 5.0. Binding was determined from optical density (OD) measurements at 405 nm with a reference wavelength of 495 nm. Data are presented as mean IC_{50} values (μM) ± SD from two independent experiments. The signal from non-specific associations was determined from the binding of biotinylated peptide in the absence of MHC molecules (I-A^d, 0.191; I-E^d, 0.091) and was subtracted from data before analysis. The maximum optical density recorded for each isotype was 1.692 and 1.035 for I-A^d and I-E^d, respectively.

possibility of a similar role for the N-terminal residues of mouse CLIP to catalyse the release of this ligand from mouse I-A molecules. Here, it was of interest to examine these residues with respect to their interaction with mouse class II MHC molecules of the I-E isotype, this time at pH 5.0.

The results from the immunoassay using truncated CLIP and I-E^d molecules (Table 5.1) did not provide any evidence of a contribution of binding energy, either positive or negative, by the native N-terminal residues of this peptide sequence. To investigate the role of N-terminal CLIP residues further, the binding of the set of frameshifted 15-mer CLIP ligands was assessed in a competitive immunoassay (Table 5.2). Data are presented as mean IC_{50} values ± standard deviation from two independent experiments and are shown in comparison to those obtained with purified I-A^d under the same conditions (these latter results have been discussed previously in *Chapter 4*, §4.3.3). Overall, those CLIP ligands that contained all or part of the original N-terminal sequence (CLIP86–100, 87–101 and CLIP88–102) were found consistently to compete better for binding to the I-E^d molecules than those that exhibited the corresponding C-terminal CLIP residues (CLIP89–103, 90–104). This is an equivalent result to that obtained for I-A^d under the same experimental conditions (Table 5.2) and for I-A^d, I-A^u and I-A^k previously, using the cell-surface peptide binding assay (*Chapter 4*, Table 4.3). Unfortunately, however, large experimental errors again made it difficult to assess the possibility that the individual N-terminal CLIP residues may mediate a self-release mechanism to free this ligand from its association with I-E^d molecules.

Next, the contribution of the outermost CLIP residues to the interaction with the I-E^d molecules was explored by evaluating the binding abilities of another set of peptide analogues, this time exhibiting a number of different length variations and/or L-alanine substitutions (Table 5.3). As before, data are presented as mean IC_{50} values ± standard deviation from two independent experiments and are shown in comparison to those obtained with purified I-A^d under the same conditions (discussed in *Chapter 4*, §4.3.3). Of particular interest, the truncation of the outermost residues of the CLIP86–104 peptide appeared to improve slightly the capacity of this sequence to bind, *e.g.* CLIP87–102 and 88–103. This result would be compatible with the native residues, Lys 86 and Pro 87, providing an inhibitory or weakening component to the binding interaction with I-E^d, as would be expected if these residues participated in a self-release mechanism like that proposed by Kropshofer *et al.* (1995a, 1995b) for HLA-DR molecules. However, large experimental error amongst the IC_{50} values for the different CLIP analogues again make it difficult to exclude the possibility that this improvement in binding may be the result of the truncation of the C-terminal residues, *e.g.* Ser 103 and Met 104.

Table 5.2 *Competition by frameshifted CLIP 15-mers for binding to affinity-purified mouse class II MHC molecules at pH 5.0.*

CLIP		IC_{50} (μM)	
		$I\text{-}A^d$	$I\text{-}E^d$
86–100	KPVSQMRMATPLLMR	91.47 ± 71.07	179.60 ± 169.17
87–101	PVSQMRMATPLLMRP	158.10 ± 85.98	126.60 ± 62.63
88–102	VSQMRMATPLLMRPM	224.70 ± 150.15	212.90 ± 162.65
89–103	SQMRMATPLLMRPMS	> 1000	> 1000
90–104	QMRMATPLLMRPMSM	> 1000	> 1000

Fifteen-residue CLIP variants were synthesised, each with a single amino acid frameshift. Analogues were tested at multiple doses as competitors against biotinylated CLIP86–104 (30 μM) for binding to the affinity-purified mouse class II MHC molecules, $I\text{-}A^d$ and $I\text{-}E^d$, in an immunoassay at pH 5.0. Binding was determined from optical density (OD) measurements at 405 nm with a reference wavelength of 495 nm. Data are presented as mean IC_{50} values (μM) ± SD from two independent experiments. Background levels and maximum signals for each isotype are as in Table 5.1.

Table 5.3 *Competition by substituted and/or length-altered CLIP for binding to affinity-purified mouse class II MHC molecules at pH 5.0.*

CLIP		IC_{50} (μM)	
		I-A^d	I-E^d
86–104	KPVSQMRMATPLLMRPMSM	91.19 ± 64.12	272.50 ± 67.15
81–104	LPKSAKPVSQMRMATPLLMRPMSM	336.80 ± 392.50	393.70 ± 198.65
81–109	LPKSAKPVSQMRMATPLLMRPMSMDNMLL	441.70 ± 231.75	434.50 ± 348.55
Ala-CLIP	AAVSQMRMATPLLMRPMAA	325.40 ± 223.15	323.30 ± 127.60
86–103	KPVSQMRMATPLLMRPMS	179.00 ± 136.17	240.80 ± 64.90
86–103A	KPVSQMRMATPLLMRPMA	219.00 ± 92.65	243.00 ± 160.85
87–104	PVSQMRMATPLLMRPMSM	347.60 ± 58.75	302.40 ± 104.50
87A–104	AVSQMRMATPLLMRPMSM	316.50 ± 352.10	381.10 ± 172.15
87–102	PVSQMRMATPLLMRPM	397.50 ± 488.90	166.20 ± 60.15
88–103	VSQMRMATPLLMRPMS	474.90 ± 1273.69	146.80 ± 82.64

Substituted and/or length-altered CLIP analogues were tested at multiple doses as competitors against biotinylated CLIP86–104 (30 μM) for binding to the affinity-purified murine class II MHC molecules, I-A^d and I-E^d, in an immunoassay at pH 5.0. Binding was determined from optical density (OD) measurements at 405 nm with a reference wavelength of 495 nm. Data are presented as mean IC_{50} values (μM) ± SD from two independent experiments. Background levels and maximum signals for each isotype are as in Table 5.1.

Lastly, the role of these N-terminal CLIP residues in the binding to I-E^d molecules was examined using a series of CLIP81–104 analogues incorporating single L-alanine substitutions of the residues, 81–87 (*Chapter 4*, Table 4.4). A marked decrease in the binding capacity, compared to wild-type CLIP81–104, was observed when either of the residues Lys 83 or Pro 87 were replaced by L-alanine (Figure 5.9a). These results closely resemble those seen for binding to I-A^d under the same conditions (*Chapter 4*, Figure 4.9b). Furthermore, when unbiotinylated variants were tested as competitors, these same substituted analogues, K83A and P87A, showed a corresponding decrease in the ability to inhibit the binding of the biotinylated wild-type peptide (Figure 5.9b). In this context, these particular native N-terminal residues appear to contribute positive binding energy to the interaction of CLIP with I-E^d MHC molecules, rather than destabilising it to facilitate peptide release.

5.3.5 The induction of CLIP binding to cell-surface I-E molecules at neutral pH

The results of both antigen-presentation and cell-surface binding assays in this chapter (Figures 5.1–5.4) have indicated that the binding of CLIP to I-E class II MHC molecules is highly sensitive to proton concentration. Specifically, an interaction between these components may be detected substantially only under conditions of low pH (Figure 5.5). This would suggest that, at neutral pH, the CLIP ligand is unable to make sufficient favourable contacts with residues of the I-E peptide-binding groove to stabilise the complex. The amino acid side chains required in a peptide sequence to enable it to bind to I-E molecules have been determined by an examination of the natural ligands of these molecules. Typically, peptides that bind to I-E class II MHC molecules show a high frequency of polar residues. These occur most commonly within the ligand at the positions corresponding to the pockets, 6 and 9, with a specific preference for basic residues at P9 (Sette *et al.*, 1989c; Marrack *et al.*, 1993; Reay *et al.*, 1994; Schild *et al.*, 1995). Most interestingly, both of these positions have been implicated previously in the pH-dependent binding of peptides to I-E $\alpha\beta$ dimers (Boniface *et al.*, 1993; Fremont *et al.*, 1996).

To test the hypothesis that CLIP is unable to bind to I-E molecules at neutral pH due to a lack of electrostatically-suitable anchors, CLIP86–104 analogues were synthesised with a single lysine residue introduced at either position, P6 (P96K), P9 (M99K) or both (P96K/M99K; Figure 5.10a). These peptides were then examined using a cell-surface binding assay for their ability to compete with the antigen-derived ligand, biotinylated

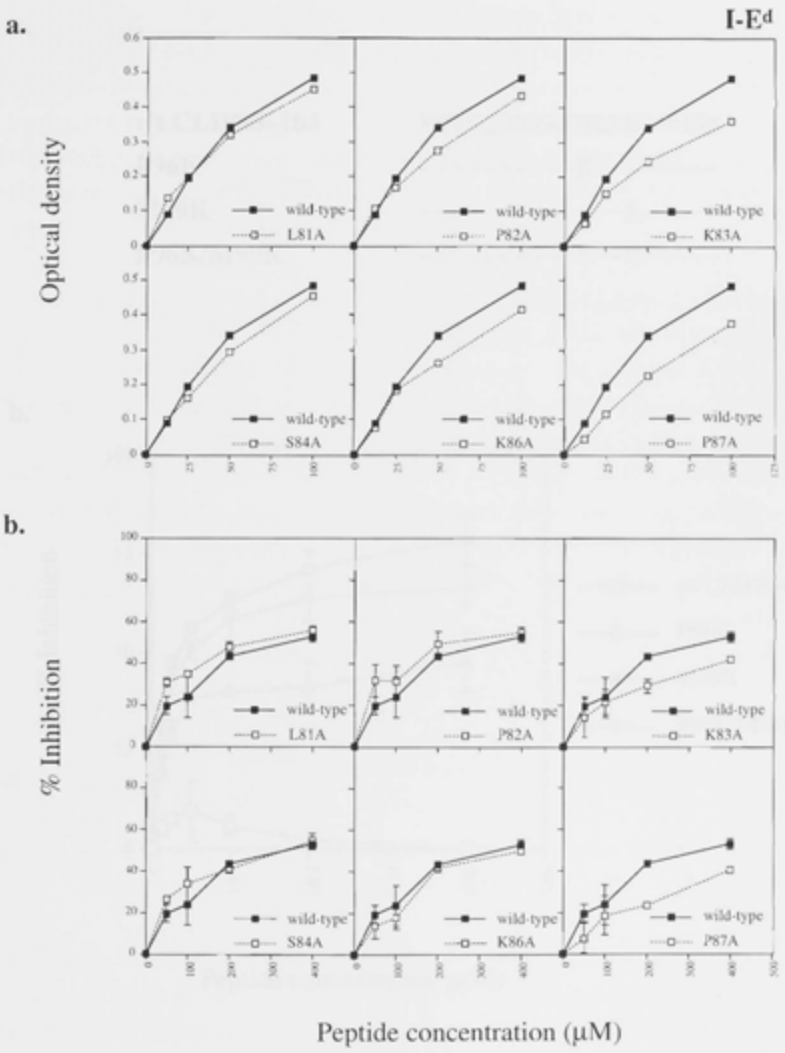


Figure 5.9 *The binding of N-terminal L-alanine-substituted CLIP81–104 to purified I-E^d in an immunoadsorption assay at pH 5.0.*

(a) Biotinylated CLIP81–104 analogues with single L-alanine substitutions in their N-terminus (dashed lines, open squares) were tested for binding to I-E^d molecules purified from the BALB/c-derived B cell lymphoma, A20. Shown in comparison with biotinylated wild-type CLIP81–104 (solid line, filled sqaures), is one of three experiments, all with equivalent results. **(b)** Unbiotinylated variants of the same analogues were tested as competitors against biotinylated wild-type CLIP81–104 (30 μM; solid line, filled squares). Shown is the mean percentage inhibition ± SD from two independent experiments. Peptides were incubated at pH 5.0 with immunopurified I-E^d for 48 hours at 37°C before capture on a M5/114-coated microtitre plate, as described in *Chapter 2*, §2.7.3. The signal from non-specific associations was determined from the binding of biotinylated peptide in the absence of MHC molecules and has been subtracted from the data shown (**a,** 0.183 OD units; **b,** 0.098 OD units).

a.

wt CLIP86–104	KPVSQMRMATPLLMRPMSM
P96K	----------K---------
M99K	----------K-----
P96K/M99K	----------K--K-----

b.

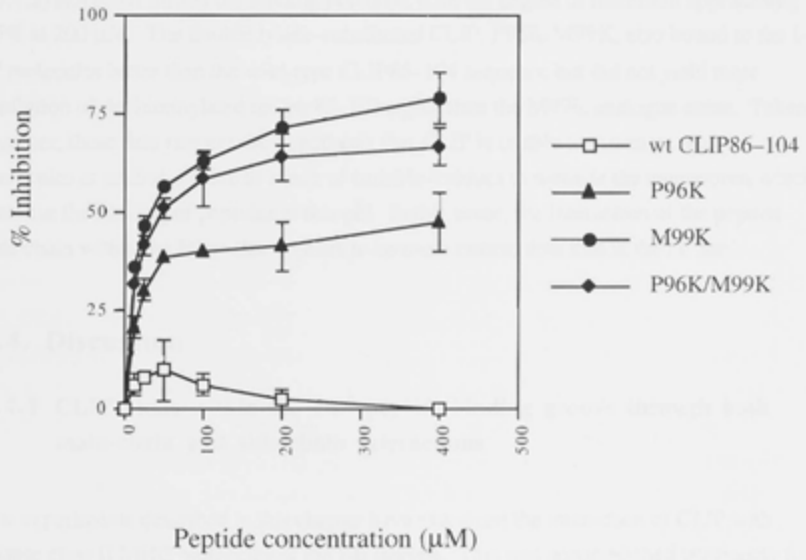


Figure 5.10 *The binding of lysine-substituted CLIP analogues to cell-surface I-E^k molecules at pH 7.0.*

Substituted CLIP analogues were examined for their ability to compete with biotinylated mCytc88–103 (75 μM) for binding to the I-E^k molecules expressed on the surface of the cell line, CH27 (H-2^k). Data are presented as the mean percentage inhibition ± SD from two independent experiments. CH27 cells were incubated with biotinylated peptide and competitor for 18 hours at 37°C before flow cytometric analysis, as described in *Chapter 2*, §2.7.2. The signal from non-specific associations was determined in the absence of biotinylated peptide and has been subtracted from the results shown.

mCytc88–103, for binding to I-E^k molecules expressed by CH27 cells (Figure 5.10b). Consistent with previous results (Figure 5.2), wild-type CLIP86–104 (filled squares) was a poor competitor against the biotinylated antigenic peptide under these conditions. Indeed, in this assay, the ability of CLIP to compete was diminished even further than in Figure 5.2 due to a higher dose of biotinylated mCytc88–103 used to generate the fluorescence signal, *i.e.* 75 μM in this instance, compared with 25μM in the previous assay.

Strikingly, however, the introduction of a lysine residue into the P6 position of the CLIP86–104 sequence (P96K, filled triangles) improved considerably its ability to compete with the biotinylated mCytc88–103 for binding to the cell-surface I-E^k molecules. A similar substitution into the CLIP sequence at the P9 position (M99K, filled circles) enhanced further the binding potential, with the degree of inhibition approaching 75% at 200 μM. The double lysine-substituted CLIP, P96K/M99K, also bound to the I-E^k molecules better than the wild-type CLIP86–104 sequence but did not yield more inhibition of the biotinylated mCytc88–103 signal than the M99K analogue alone. Taken together, these data support the hypothesis that CLIP is unable to associate with I-E molecules at neutral pH due to a lack of suitable residues to mediate the interactions which stabilise the binding of peptides at this pH. In this sense, the interaction of the peptide side chain within the P9 pocket appears to be more critical than that at the P6 site.

5.4. Discussion

5.4.1 CLIP binds within the I-E^d peptide-binding groove through both main-chain and side-chain interactions

The experiments described in this chapter have examined the interaction of CLIP with mouse class II MHC molecules of the I-E isotype. This was accomplished previously for I-A molecules by assessing the binding of different substituted and/or truncated CLIP analogues in both antigen-presentation and cell-surface peptide binding assays (*Chapters 3 & 4*). However, unlike their I-A counterparts, I-E class II MHC molecules were found to bind CLIP ligands poorly using these same assay methods (Figures 5.1–5.4). This was consistent with observations in *Chapters 3* (§3.3.1) & *4* (§4.3.1) of an apparently negligible contribution from biotinylated CLIP binding to I-E heterodimers in cell-surface binding assays with the CH27 (H-2^k) and A20 (H-2^d) cell lines.

The formation of most peptide–class II MHC complexes has been noted to be augmented at low pH (*e.g.* Jensen, 1990, 1991; Harding *et al.*, 1991; Wettstein *et al.*, 1991; Mouritsen *et al.*, 1992; Sette *et al.*, 1992b). Thus, in an effort to find a better means with which to assess the association of CLIP with I-E molecules, the effect of proton concentration upon this interaction was examined in an immunoassay with purified I-E^d (Figure 5.5). At neutral pH, CLIP bound to the I-E^d molecules with low affinity. This result is consistent with the findings of the cell-surface and antigen-presentation assays (Figures 5.1–5.4) which were conducted with whole cells in media buffered by HEPES (pH 7.2–8.2; *Appendix B1*). By contrast, the binding of CLIP to I-E molecules was enhanced at pH 5.0 to the extent that the presence of such complexes could now be measured experimentally (Figure 5.5). Based on these findings, a change in the methodology used to examine the CLIP–I-E interaction was implemented, such that all subsequent experiments were conducted using purified I-E molecules in an enzyme-linked immunoassay at pH 5.0.

Using this approach to examine the binding of the truncated and/or substituted CLIP analogues to purified I-E^d, the features of CLIP that are important for interacting with these class II MHC molecules have been elucidated. Firstly, an assessment of the binding of D-alanine-substituted CLIP86–104 analogues to I-E^d identified a critical role for the main-chain atoms of the central sequence element, CLIP90–99, in providing positive binding energy to stabilise this interaction (Figure 5.7). Secondly, within this region of the CLIP sequence, the substitution by L-alanine of any of the side chains, Met 91, Thr 95, Leu 98 or Met 102 eliminated the capacity of CLIP to bind to the I-E^d heterodimer. The L-alanine substitution of Met 99 also diminished the interaction, whilst the equivalent substitution of the Pro 96 residue led to an increase in binding capacity (Figure 5.8). Meanwhile, the assessment of the binding of truncated CLIP variants indicated that for efficient association of this sequence with the I-E^d molecules, these residues must be incorporated into a peptide of more than 13 residues in length (Table 5.1). The binding of CLIP81–104 analogues exhibiting single L-alanine substitutions within the N-terminal sequence revealed that the conserved residues, Lys 83, Pro 87 and to a lesser extent, Lys 86, also appeared to provide a favourable influence on the interaction of the CLIP ligand with I-E^d molecules (Figure 5.9). Lastly, strong binding of CLIP to I-E^k molecules at neutral pH was enabled by introducing lysine residues into either position, P6 (P96K) or P9 (M99K), within the peptide sequence (Figure 5.10).

In order to interpret the experimental findings reported in this chapter regarding the mode of CLIP binding to I-E^d, it is necessary to have an understanding of how peptides associate typically with these molecules. Such details may now be extrapolated from the

X-ray crystal structures of the I-E^k αβ dimer complexed with either the mouse haemoglobin peptide, mHb64–76, or a determinant from the mouse heat shock protein 70, mHsp236–248 (Fremont *et al.*, 1996). It may be assumed that I-E^d molecules are likely to share a high degree of structural homology with I-E^k given the slight divergence between their primary sequences, as characteristic of all class II MHC molecules of this isotype. For example, a comparison of the α-chain sequences of the I-E^d and I-E^k allotypes reveals just two amino acids differences over the entire protein, with these being restricted one each to the second domain and cytoplasmic tail (Ayane *et al.*, 1986; for a description of the domain organisation of class II MHC molecules, see *Chapter 1*, Figure 1.3). Additionally, the two molecules also exhibit 91% sequence identity between their respective Eβ-chains (Mengle-Gaw & McDevitt, 1983).

The X-ray crystallographic analysis of the I-E^k αβ dimer (Fremont *et al.*, 1996) revealed that peptides bind to these molecules in the same manner as ligands complexed with human HLA-DR (Brown *et al.*, 1993; Stern *et al.*, 1994; Ghosh *et al.*, 1995) and mouse I-A molecules (Fremont *et al.*, 1998b; Scott *et al.*, 1998). Specifically, a nine-residue stretch of the peptide is encompassed within the peptide-binding groove, being tethered by both a comprehensive network of hydrogen bonds between the main-chain atoms of the peptide and the class II MHC residues, and interactions between the peptide side chains and the polymorphic class II MHC pockets. Furthermore, the I-E^k heterodimer exhibits the same location and spacing of the five major pockets observed in other crystal structures and predicted in homology models of HLA-DR and I-A molecules, corresponding to the relative positions within the bound peptide at P1, P4, P6, P7 and P9, where P1 represents the first amino acid within the peptide N-terminus to be encompassed fully within the peptide-binding groove (Stern *et al.*, 1994; Ghosh *et al.*, 1995; Lee & McConnell, 1995; Fremont *et al.*, 1996, 1998b; Dessen *et al.*, 1997; Lee *et al.*, 1998; Scott *et al.*, 1998; Weber *et al.*, 1998). The arrangement of these pockets had been predicted previously for both I-E^k and I-E^d molecules based upon the results of substitution and truncation analyses of a range of different I-E-binding peptides, together with pool sequencing of natural ligands eluted from these molecules (Marrack *et al.*, 1993; Reay *et al.*, 1994; Schild *et al.*, 1995).

Some details of the manner in which I-E class II MHC molecules bind CLIP may also be extrapolated from knowledge of how this sequence interacts with HLA-DR heterodimers since these molecules represent the human homologue of I-E, *i.e.* they too exhibit minimal variation in their α-chain sequence (*e.g.* Korman *et al.*, 1982; Larhammar *et al.*, 1982; Lee *et al.*, 1982; Das *et al.*, 1983). The I-E and HLA-DR α-chains also display a high degree of amino acid sequence identity, as do the I-E and HLA-DR β-chains,

excluding the hypervariable segments (Allison *et al.*, 1978; Silver *et al.*, 1979). For example, the $E\alpha_1$ domain shows 79% sequence homology at the protein level to its human counterpart, HLA-DRα_1 (McNicholas *et al.*, 1982; for a complete alignment of the deduced amino acid sequences of mouse and human class II MHC molecules, see Figueroa & Klein, 1986 and Klein, 1986). By contrast, the α_1 and β_1 domains of I-E^k and I-A^k molecules show just 55% and 62% sequence identity, respectively (Benoist *et al.*, 1983b; Mengle-Gaw & McDevitt, 1983).

In the context of details provided by the peptide–I-E^k crystal structures (Fremont *et al.*, 1996) and data from human CLIP crystallised in association with HLA-DR3 (Ghosh *et al.*, 1995), the results presented herein regarding the binding of mouse CLIP to I-E^d class II MHC molecules indicate that positive binding energy is provided by both the formation of a hydrogen-bond network through the peptide backbone (Figure 5.7) and *via* several peptide side chains forming anchor interactions with the residues of the $\alpha\beta$ dimer (Figure 5.8). This latter feature is particularly noteworthy given that CLIP does not make significant anchor contacts through its side chains when binding to variants of the mouse I-A isotype, with the interaction instead being sustained predominantly by the main-chain hydrogen-bonding scheme (*Chapter 3*, §3.4.2 & *Chapter 4*, §4.4.1).

5.4.2 An examination of specific CLIP side-chain interactions with pockets of the I-E^d peptide-binding groove

Further insight into the molecular basis of the favourable interactions occurring at pH 5.0 between the I-E^d molecules and the CLIP residues, Met 91, Thr 95, Leu 98, Met 99 and Met 102, may be gained from an examination of the particular sites upon the $\alpha\beta$ dimer at which these side chains interact. This may be accomplished by comparing known I-E^d-specific sequence polymorphism with details of the I-E^k crystal structures (Fremont *et al.*, 1996) and relating the points of allelic variation to the peptide-binding preferences of these two MHC molecules. Despite the observation that I-E variants have a high degree of sequence conservation overall, such comparisons remain important because localised areas of polymorphism still exist within the β_1 domains, affecting the character of the pockets within the peptide-binding groove. For example, the I-E^d and I-E^k allotypes exhibit just 78% protein sequence identity within this region (Mengle-Gaw & McDevitt, 1983). Similar comparisons were performed in *Chapter 3* (§3.4.4) to elucidate how CLIP side-chains interact with I-A $\alpha\beta$ dimers. For I-E molecules, additional information on this matter may also be provided from knowledge of how peptides interact with the homologous human molecules, HLA-DR.

A summary of the interactions made by CLIP side chains within the pockets of the I-E^d class II MHC peptide-binding groove is shown in Table 5.4, based upon the experimental results presented herein (Figure 5.8), together with data from X-ray crystallographic studies of I-E^k and other αβ dimers (Stern *et al.*, 1994; Ghosh *et al.*, 1995; Fremont *et al.*, 1996, 1998b; Dessen *et al.*, 1997; Scott *et al.*, 1998), homology models (Bogen & Lambris, 1989; Sette *et al.*, 1989a; Chu *et al.*, 1994; Gregori *et al.*, 1999), sequence comparisons (Benoist *et al.*, 1983b; Mengle-Gaw & McDevitt, 1983; Ayane *et al.*, 1986; Chu *et al.*, 1994; Fremont *et al.*, 1998b) and analyses of the peptide-binding motifs of different I-E allotypes (Guillet *et al.*, 1987; Adorini *et al.*, 1988a; Bogen & Lambris, 1989; Sette *et al.*, 1989a, 1989b, 1989c; Evavold *et al.*, 1992; Boniface *et al.*, 1993; Marrack *et al.*, 1993; Reay *et al.*, 1994; Rammensee *et al.*, 1995; Schild *et al.*, 1995; Gregori *et al.*, 1999). Using the same approach, predictions are also given for how these CLIP residues might interact with I-E^k αβ dimers.

The nature of the CLIP–I-E interaction may be inferred by extrapolating from details of the X-ray crystallographic analysis of human CLIP complexed with HLA-DR3 (Ghosh *et al.*, 1995). It may be anticipated that mouse CLIP would bind with the Met 91 anchor residue accommodated within the first major pocket, at relative position P1. This binding register is confirmed by the findings reported herein, whereby the central sequence element of CLIP, residues 90–99, is critical for sustaining an interaction with the I-E^d molecule (Figure 5.7). The same alignment is also adopted when this ligand binds to different I-A allotypes (*Chapter 3*, §3.4.4; Lee & McConnell, 1995).

In common with HLA-DR molecules, the P1 pocket of I-E αβ dimers is lined with a number of conserved, hydrophobic residues (Brown *et al.*, 1993; Stern *et al.*, 1994; Ghosh *et al.*, 1995; Fremont *et al.*, 1996; Dessen *et al.*, 1997). The exact side-chain dimensions able to be accommodated within this pocket in both HLA-DR and I-E are dictated by the nature of the residue at the polymorphic position 86 in the β-chain of the heterodimer (Busch *et al.*, 1991; Demotz *et al.*, 1993; Newton-Nash & Eckels, 1993; Verreck *et al.*, 1993; Fremont *et al.*, 1996). In I-E^d, the P1 pocket is large as a result of the β86 position being occupied by a small serine residue (Mengle-Gaw & McDevitt, 1983). This situation is equivalent to presence of glycine at this position in HLA-DR1 (discussed previously in *Chapter 3*, §3.4.5; Stern *et al.*, 1994). Accordingly, pool sequencing of natural I-E^d peptide ligands has revealed a predominance of the bulky aromatic residues, tyrosine, tryptophan and phenylalanine at relative position, P1 (Schild *et al.*, 1995). The requirement for the Met 91 CLIP side chain to act as an anchor for the binding interaction with I-E^d thus lies in the capacity of its side chain to make strongly favourable hydrophobic contacts within this pocket (Figure 5.8). It is interesting to note

Table 5.4 *The interaction of CLIP side chains with the pockets of the I-E peptide-binding groove.*

Shown are the amino acid residues whose atoms contribute to forming the major polymorphic pockets of the peptide-binding grooves of the class II MHC molecules, I-E^d and I-E^k. Where data are available, these residues have been taken from the X-ray crystallographic analysis of the I-E^k αβ dimer complexed with either of the antigen-derived peptides, murine heat shock protein 70, mHsp236–248, or murine haemoglobin, mHb64–76 (Fremont *et al.*, 1996). Alternatively, residues have been assigned by equivalent analyses of allelic variants of the human homologue, HLA-DR, together with I-E homology models (Bogen & Lambris, 1989; Sette *et al.*, 1989a; Chu *et al.*, 1994; Stern *et al.*, 1994; Ghosh *et al.*, 1995; Gregori *et al.*, 1999). Within each pocket, these residues form either hydrogen bonds or van der Waals interactions with the atoms of the amino acid side chains of a bound peptide. Throughout this thesis, class II MHC residues are numbered according to the secondary structure-based sequence alignment of the α_1 and β_1 domains of the molecules, I-E^k, I-A^k and HLA-DR, reported by Fremont *et al.* (1998b). The primary amino acid sequences of the α_1 and β_1 domains of the different I-E molecules are taken from Benoist *et al.* (1983), Mengle-Gaw & McDevitt (1983), Ayane *et al.* (1986) and Chu *et al.* (1994). Polymorphic residues between the I-E molecules are italicised. Those underlined provide a specific physicochemical property to the pocket to govern its overall peptide side-chain preference. For each I-E allotype, the peptide residues commonly found at each of these positions have been taken from alignments of naturally-processed ligands (Guillet *et al.*, 1987; Adorini *et al.*, 1988a; Bogen & Lambris, 1989; Sette *et al.*, 1989a, 1989b, 1989c; Evavold *et al.*, 1992; Boniface *et al.*, 1993; Marrack *et al.*, 1993; Reay *et al.*, 1994; Rammensee *et al.*, 1995; Schild *et al.*, 1995; Gregori *et al.*, 1999). The individual side chains of the native CLIP sequence that bind at these positions are shown in italics beneath the respective pocket number.

[a]The nature of the interaction of the native CLIP side chains with I-E^d molecules is summarised from the results of the immunoassay at pH 5.0 presented in this chapter (Figure 5.8).

[b]Predictions are also given for how these CLIP residues might interact with I-E^k αβ dimers, based upon the data listed above.

[c]βAsp 57 is mutated to a serine residue in the I-A^{g7} class II MHC molecule of the non-obese diabetic (NOD) mouse. The presence of a non-Asp residue at this position has been linked to the development of autoimmune diabetes in both these animals and in humans. The effects of this substitution on the peptide-binding properties of the I-A^{g7} molecule are examined in *Chapter 6* of this thesis.

⚓ represents an anchor side chain within the CLIP sequence, such that binding to the I-E molecule is not sustained if this residue is substituted with L-alanine.

✓ represents the provision of positive binding energy by this CLIP side chain at the binding interface.

↯ represents the provision of negative binding energy by this CLIP side chain at the binding interface.

— represents no notable contribution of binding energy by the CLIP side chain to the interaction with I-E or, in the case of the wild-type CLIP residue at the P4 position, Ala 94, where this side chain was not examined by substitution analysis.

Allotype	P1 *Met 91*	P4 *Ala 94*	P6 *Pro 96*	P7 *Leu 97*	P9 *Met 99*
I-E^d	αIle 7, αIle 31, αPhe 32, αVal 34, αTrp 43, βIle 85, *βSer 86*, βPhe 89, βLeu 90	αGln 9, βSer 13, *βAsp 70*, *βAla 71*, *βSer 74*, *βTyr 78*	αGlu 11, αAsn 62, αVal 65, αAsp 66, *βVal 11*, βSer 13	αAsn 69, *βGlu 28*, *βTyr 47*, βTrp 61, βPro 65, *βIle 67*, *βAsp 70*, *βAla 71*	αAsn 69, αVal 72, αMet 73, αArg 76, βGlu 9, βAsp 57[c]
Pocket character:	large, hydrophobic	medium, acidic	medium, hydrophobic	shallow, solvent-exposed	narrow, hydrophobic
Residues observed:	*e.g.* Tyr, Trp, Phe	*e.g.* Lys, Arg	*e.g.* Ile, Val, Leu	*e.g.* Lys, Arg	*e.g.* Lys, Arg
[a]*CLIP interaction:*	⚓	—	↯	—	✓
I-E^k	αIle 7, αIle 31, αPhe 32, αVal 34, αTrp 43, βIle 85, *βPhe 86*, βPhe 89, βLeu 90	αGln 9, βSer 13, *βGln 70*, *βLys 71*, *βGlu 74*, *βVal 78*	αGlu 11, αAsn 62, αVal 65, αAsp 66, *βCys 11*, βSer 13	αAsn 69, *βVal 28*, *βPhe 47*, βTrp 61, βPro 65, *βPhe 67*, *βGln 70*, *βLys 71*	αAsn 69, αVal 72, αMet 73, αArg 76, βGlu 9, βAsp 57[c]
Pocket character:	medium, hydrophobic	large, amphiphilic	medium, hydrophilic	shallow, solvent-exposed	narrow, hydrophobic
Residues observed:	*e.g.* Ile, Leu, Val	*e.g.* Ser, Val, Ile, Leu, Phe	*e.g.* Gln, Asn, Asp, Glu	degenerate	*e.g.* Lys, Arg
[b]*CLIP interaction:*	⚓	—	↯	—	✓

that the presence of an anchor residue at the P1 position is not typically a stringent requirement for a peptide to be able to bind to molecules of the I-E^d allotype, presumably due to more important interactions taking place elsewhere within the groove (see below; Sette *et al.*, 1989a). By contrast, this position represents a primary anchor site for natural ligands of I-E^k, such that the substitution of an amino acid here reduces binding dramatically (Leighton *et al.*, 1991; Reay *et al.*, 1994). In this respect, an anchor interaction may also be predicted between the Met 91 residue of the CLIP sequence and the hydrophobic P1 pocket of I-E^k molecules. Aliphatic residues are most common within natural ligands here as a result of the larger phenylalanine residue at β86 (Mengle-Gaw & McDevitt, 1983; Marrack *et al.*, 1993; Schild *et al.*, 1995; Fremont *et al.*, 1996). For example, the P1 pocket accommodates a valine or isoleucine side chain in the mHsp–I-E^k and mHb–I-E^k crystal structures, respectively (Fremont *et al.*, 1996). This effect is similar to that seen when valine occupies the β86 position, as in HLA-DR3 (discussed previously in *Chapter 3*, §3.4.5; Ghosh *et al.*, 1995; Dessen *et al.*, 1997).

The P4 pocket within the I-E peptide-binding groove is even larger than that accommodating the P1 side chain, as revealed in the X-ray crystallographic analysis of I-E^k complexed with the mHb64–76 and mHsp236–248 peptides (Fremont *et al.*, 1996). The role of the native CLIP residue that corresponds to this site was not examined by substitution analysis in this study, being already L-alanine (Ala 94). However, such a small side chain is unlikely to contribute significant binding energy at this site, either positive or negative. Similar to HLA-DR molecules, the P4 pocket of I-E molecules is lined with a greater number of polymorphic residues than the P1 pocket which results in more marked allele-specific side-chain preferences at this position (Fremont *et al.*, 1996; Stern *et al.*, 1994; Ghosh *et al.*, 1995; Rammensee *et al.*, 1995; Chelvanayagam, 1997; Dessen *et al.*, 1997; Gregori *et al.*, 1999). Between the I-E^d and I-E^k αβ dimers, the most important of these sequence variations involve the residues β70, β71, β74 and β78 (Table 5.4; Mengle-Gaw & McDevitt, 1983). Consequently, the P4 pocket of I-E^k displays a preference for either aliphatic or polar side chains, with serine, valine and isoleucine residues being common in natural peptide ligands (Schild *et al.*, 1995). Large aromatic residues may also be accommodated within this site, as indicated by the presence of phenylalanine in the P4 pocket of both peptide–I-E^k crystal structures (Fremont *et al.*, 1996). By contrast, this site within I-E^d class II MHC molecules is more electrostatically-negative in character due to the aspartic acid residue at position, β70 (Mengle-Gaw & McDevitt, 1983) and, consequently, basic residues are found frequently at this position in both I-E^d-binding peptides and natural ligands (Schild *et al.*, 1995).

The P6 pocket of I-E and HLA-DR heterodimers also shows significant amino acid polymorphism between allotypes (Hammer *et al.*, 1993; Rammensee *et al.*, 1995; Chelvanayagam, 1997; Gregori *et al.*, 1999). Two of the key residues involved in imparting chemical character to this site are located at positions β11 and β13 (Ghosh *et al.*, 1995; Dessen *et al.*, 1997; Fremont *et al.*, 1996). In the I-E^k molecule, these sites are occupied by the polar residues, cysteine and serine, respectively (Mengle-Gaw & McDevitt, 1983). Accordingly, this pocket accommodates the polar side chain of Glu 73 in the mHb–I-E^k crystal structure and Ala 243 plus several water molecules in the mHsp–I-E^k complex (Fremont *et al.*, 1996). From pool sequencing data and other analyses of I-E^k binding motifs, asparagine and glutamine residues are also commonly found at the P6 position (Marrack *et al.*, 1993; Reay *et al.*, 1994; Schild *et al.*, 1995). By contrast, this pocket within I-E^d molecules is more hydrophobic with a specific preference for aliphatic side chains (Schild *et al.*, 1995). Nevertheless, the pyrrolidine ring of the CLIP Pro 96 residue is inhibitory to binding at this site, presumably due to steric hindrance (Figure 5.8). A similar situation would also be predicted for this residue interacting at the P6 pocket of the I-E^k molecule.

The P7 pocket of most class II MHC molecules is degenerate and able to accommodate a diverse range of different amino acid side chains, as described previously in *Chapter 3* (§3.4.4). This is likely to be a consequence of this site being shallow, with the incoming peptide side chain oriented towards the α-helical walls of the cleft rather than downwards towards the floor (Stern *et al.*, 1994; Ghosh *et al.*, 1995). The P7 pocket of I-E^k molecules would appear to be no exception and the side chain of the Glu 244 residue that interacts at this position in the mHsp–I-E^k crystal structure shows high solvent exposure (Fremont *et al.*, 1996). Consistent with this arrangement, P7 side chains in peptide–I-E^k complexes form contacts with the αβ dimer (Evavold *et al.*, 1992; Driscoll *et al.*, 1993) but may also interact with the TCR (Bhayani & Paterson, 1989; Leighton *et al.*, 1991; Reay *et al.*, 1994; Hsu *et al.*, 1996). It might be expected that I-E^d molecules would show a similar lack of preference for the type of amino acid side chain that interacts within the P7 pocket since they share most of the MHC residues that line this site in I-E^k (Fremont *et al.*, 1996). However, pool sequencing and the alignment of natural ligands eluted from I-E^d molecules have revealed that positively-charged residues are frequently accommodated at this position (Sette *et al.*, 1989a; Schild *et al.*, 1995). In this respect, it has been suggested that a basic side chain at P7 may form a salt-bridge with either βGlu 28 or the nearby βAsp 70 of the I-E^d heterodimer (Bogen & Lambris, 1989; Sette *et al.*, 1989a; Leighton *et al.*, 1991; positions β114 and β155, respectively, according to the HLA-A2 numbering used by these authors), this latter residue also having been proposed to contribute to the electrostatically-negative character of the P4 pocket (Schild *et al.*,

1995). Both of these positions are occupied by uncharged amino acids in I-E^k (Mengle-Gaw & McDevitt, 1983). In this study, little difference was seen when the native CLIP residue, Leu 97, was substituted with L-alanine but neither side chain provides an assessment of charge discrimination (Figure 5.8).

Lastly, the P9 pockets of all I-E class II MHC allotypes examined to date exhibit an identical preference for a positively-charged side chain (Marrack *et al.*, 1993; Reay *et al.*, 1994; Schild *et al.*, 1995; Gregori *et al.*, 1999). This specificity arises from the ability of such a residue to provide considerable positive binding energy to the interaction by forming a salt bridge with the I-E-conserved amino acid, βGlu 9. In both peptide–I-E^k crystal structures, this residue lies at the base of a deep cavity which is otherwise predominantly hydrophobic along its length. Thus, the native CLIP residue, Met 99, is more favoured for binding to I-E^d than L-alanine at this position due to the ability of its side chain to participate in some of these hydrophobic interactions (Figure 5.8). Nevertheless, the lack of a positive charge to stabilise this complex further leaves it extremely sensitive to proton concentration, such that CLIP is unable to associate with both I-E^d and I-E^k molecules at neutral pH (Figures 5.1–5.4). Accordingly, when a lysine residue is introduced into the CLIP sequence (M99K), this ligand may now bind to I-E^k molecules on the surface of CH27 cells at pH 7.0 (Figure 5.10). The pH dependence of the binding of CLIP to I-E class II MHC molecules is discussed further in §5.4.4.

By comparison with the crystal structures of peptide–I-E^k complexes and similar analyses of HLA-DR and I-A molecules, the remaining CLIP residues at relative positions, P2, P3, P5 and P8 are all predicted to be oriented across or away from the I-E peptide-binding groove (Stern *et al.*, 1994; Ghosh *et al.*, 1995; Fremont *et al.*, 1996, 1998b; Dessen *et al.*, 1997; Scott *et al.*, 1998). Indeed, for both I-E^k and I-E^d molecules, the side chains at these positions in antigen-derived peptides typically represent important contact sites for T cell recognition (Leighton *et al.*, 1991; Evavold *et al.*, 1992; Hsu *et al.*, 1996). Therefore, it was somewhat unusual to find that the CLIP residues Thr 95 (P5) and Leu 98 (P8) were able to form anchor associations with the I-E^d αβ dimer (Figure 5.8). Likewise, the Met 102 residue of CLIP (P12) appears to be an anchor in the interaction with I-E^d (Figure 5.8), yet X-ray crystallographic analysis would predict it to lie outside of the groove. The orientation of all these residues makes it very difficult to elucidate the molecular basis of these observations. Of particular interest, a similar situation was noted for these same CLIP residues in the binding of this sequence to mouse class II MHC molecules of the I-A isotype in *Chapter 3* (§3.4.4). In that case, it was suggested that these favourable interactions at unconventional sites were an adaptive strategy to uphold

the binding of this ligand to the αβ dimer in the absence of suitable residues within the major pockets of the groove. Regarding the peptide-binding motif of I-E^d, CLIP is indeed again lacking in many of the preferred anchor residues. For example, a key feature of the ligands of these molecules is the presence of positively-charged residues at relative positions P4, P7 and P9 (Table 5.4; Bogen & Lambris, 1989; Sette *et al.*, 1989a, 1989b, 1989c; Schild *et al.*, 1995). The CLIP sequence exhibits basic residues only at positions, P2 (Arg 92) and P10 (Arg 100), both unsuitable for alignment with the appropriate negatively-charged pockets.

The ability of the Thr 95 CLIP residue to form an anchor interaction with the I-E^d αβ dimer may be related to the presence of the insignificant side chain of Ala 94 at position P4. Rammensee *et al.* (1995) have reported that some class II MHC molecules may exhibit slightly different spacings of anchor residues and incorporate the second peptide anchor, or simply additional bonds, at the P3 or P5 positions when an unsuitable residue is present at P4. Such interactions were not specifically noted for molecules of the I-E isotype from the alignment of known peptide ligands (Rammensee *et al.*, 1995) but nor did such an approach predict the capacity of the human CLIP residue, Met 93, to form an anchor interaction with HLA-DR3 molecules, as has now been shown (discussed previously in *Chapter 3*, §3.4.4 & §3.4.5). It is interesting to note that within the crystal structure of the mHb64–76 peptide complexed with I-E^k, the side chain of the P5 residue, Asn 72, may project from the groove to become a TCR contact site but it also interacts on the surface of the protein with βGln 70, a residue of significance in the P4 and P7 pockets also (Fremont *et al.*, 1996). This position in the I-E^d β-chain is occupied by aspartic acid (Mengle-Gaw & McDevitt, 1983). Further experimentation is needed to determine whether an electrostatic contact also exists between the CLIP P5 residue, Thr 95, and βAsp 70 in the I-E^d molecule.

A similar mechanism may be proposed to account for the ability of the CLIP P8 residue, Leu 98, to form an interaction with I-E^d. For example, Schild *et al.* (1995) have observed that the peptide residue that interacts within the last major pocket of the I-E^d groove is not always located at relative peptide position, P9, but is sometimes at P8. This occurs typically when a positively-charged residue is located at P8. However, such an arrangement for CLIP binding to I-E^d is not compatible with the significant effects seen when the Met 99 residue was substituted by L-alanine (Figure 5.8). Meanwhile for I-E^k molecules, the amino acid side chain that interacts with the last pocket of the groove may sometimes be located at P10, instead of P9 (Schild *et al.*, 1995). In this case, the mouse CLIP sequence does indeed exhibit a suitable basic residue at this position, Arg 100. Nevertheless, sufficient positive binding energy was still not present in the interaction

between CLIP and I-E^k molecules to facilitate the formation of such complexes at neutral pH and the substitution of Arg 100 by L-alanine had little effect upon binding at pH 5.0 (Figure 5.8). In this respect, it is possible that the binding of a P10 lysine residue within the last pocket of the I-E^k groove may produce a weaker salt bridge than the conventional interaction through a side chain at P9. For example, the pigeon cytochrome *c* peptide, pCytc81–104, exhibits an alanine insertion that shifts the groove-terminal (P9) Lys 103 residue to the P10 position relative to the moth cytochrome *c* sequence. An examination of the binding kinetics of these peptides has revealed that the pigeon cytochrome *c* peptide dissociated faster from I-E^k at pH 7.5 (Witt & McConnell, 1991), similar to the result observed when the P9 lysine of the mCytc peptide was substituted with alanine (Boniface *et al.*, 1993). The effect of pH upon the binding of peptides to I-E class II MHC molecules is discussed further in §5.4.4.

5.4.3 CLIP binds differently to I-A and I-E class II MHC molecules

The binding of CLIP to I-E^d and I-A molecules differs significantly in the role of side-chain anchors. Such contrasting modes of binding might be predictable given the presence of similar essential contacts between CLIP and HLA-DR variants but not between this ligand and αβ dimers of the HLA-DQ isotype (these molecules represent the human homologues of I-E and I-A, respectively, as discussed previously in *Chapter 3*, §3.4.5; Geluk *et al.*, 1995; Ghosh *et al.*, 1995; Malcherek *et al.*, 1995). The ability to detect these differences in the mechanism of peptide binding between I-A and I-E molecules serves to illustrate the utility of employing the CLIP sequence to probe the peptide-binding interactions of individual class II MHC variants.

When the side chains of antigen-derived ligands form anchor contacts with class II MHC molecules, these typically take place within the pockets of the peptide-binding groove where the local environment is subject to allelic hypervariability. Thus, the formation of productive anchor contacts between CLIP side chains and the residues of I-E^d makes it interesting to consider how this ligand is able to bind promiscuously to molecules of this isotype. Consolidating the details provided in §5.4.2, this would appear to be facilitated by the fact that only one of these interactions actually takes place by conventional means within a pocket of the groove, *i.e.* Met 91 at P1. Of further significance, this pocket is atypical in having a high degree of sequence conservation between I-E allotypes (Fremont *et al.*, 1996). Meanwhile, the remaining anchor residues, Thr 95, Leu 98 and Met 102, would appear to interact with I-E^d elsewhere on the αβ dimer where the amino acid sequence is less polymorphic. For example, extrapolating from HLA-DR molecules, the

immediate environment of the P8 side chain, Leu 98, is predicted to be relatively conserved between allelic I-E variants (Chelvanayagam, 1997). In other words, none of the interactions detected in the association of CLIP with $I\text{-}E^d$ are limited to this allotype but, rather, have the potential to form when this ligand binds to any I-E variant.

A significant feature in the degenerate binding of CLIP to I-E molecules is the presence of the non-polymorphic α-chain (Ayane *et al.*, 1986; Mengle-Gaw & McDevitt, 1985), as with HLA-DR molecules (*Chapter 3*, §3.4.5). Since the antigen-binding site is composed equally of residues from both the α- and β-chains, the sequence conservation with the α_1 domain reduces the degree of variation between the individual allelic variants of this isotype considerably. By contrast, I-A molecules exhibit greater amino acid polymorphism in and around the binding groove (reviewed by Mengle-Gaw & McDevitt, 1985) and conserved anchor contacts are less able to form (*Chapter 3*; Gautam *et al.*, 1995). The non-polymorphic α-chain within HLA-DR and I-E molecules also contributes to the degenerate binding of antigen-derived ligands, as illustrated by the frequency with which allelic variants within these isotypes share the same peptide side-chain preferences, *e.g.* the common preference for a hydrophobic side chain at P1 (Table 5.4; Hammer *et al.*, 1993; Rammensee *et al.*, 1995; Schild *et al.*, 1995; Gregori *et al.*, 1999). Such effects are again less prevalent in the peptide-binding motifs of different I-A class II MHC molecules (*Chapter 3*, Table 3.2; Reizis *et al.*, 1998). The presence of conserved peptide anchor preferences between different HLA-DR molecules has been exploited to engineer a set of ligands which exhibit high-affinity binding to a broad range of HLA-DR molecules and are potent immunogens (Alexander *et al.*, 1994). These authors have proposed that such ligands may be used to generate T cell help in a variety of vaccine applications.

Nevertheless, the additional contacts made with the I-E peptide-binding groove through the CLIP side chains do not appear to add significantly to the binding energy of this interaction. For example, similar dissociation constants have been determined for CLIP-bound I-A and I-E class II MHC molecules at pH 5.5 (discussed further in §5.4.4; Bangia & Watts, 1995). Moreover, this ligand also forms side-chain interactions with HLA-DR3 αβ dimers (Ghosh *et al.*, 1995) yet these complexes remain unable to withstand dissociation of their subunits induced in the presence of SDS detergent (*e.g.* Riberdy *et al.*, 1992; Sette *et al.*, 1992a; Hitzel & Koch, 1996). Presumably a greater fulfilment of the specific pocket preferences of the $I\text{-}E^d$ αβ dimer than that provided by CLIP is still needed to provide sufficient binding energy to undergo the conformational change into the compact state. In this respect, it may be worthwhile to examine whether

greater stability in SDS detergent may be observed for I-E complexes with the CLIP analogue, M99K.

Lastly, it is interesting to consider how the high degree of sequence conservation within the α-chains of I-E and HLA-DR may affect antigen presentation by these class II MHC molecules. Such a feature is not functionally neutral, as shown by the significant effects upon the ability of I-E molecules to bind peptides and stimulate T cells when amino acid substitutions were introduced into the α-chain at positions equivalent to polymorphic sites in the I-A α-chain (Chu *et al.*, 1994). The possible roles that have been proposed for this lack of polymorphism include shaping a unique T cell repertoire and enabling a particular array of ligands to be bound and presented. However, both of these theories appear to have more experimental evidence against them than support (appraised by Chu *et al.*, 1994). Certainly, the presence of a non-polymorphic α-chain within molecules of the I-E and HLA-DR isotypes does have positive effects on the ability of peptides to bind degenerately (reviewed by Rothbard & Gefter, 1991). With regards to the interaction with CLIP, this may be important in controlling the site and timing within the endosomal compartments at which these molecules associate with antigen-derived peptides.

5.4.4 The pH dependence of CLIP binding to I-E

In this study, a significant effect of proton concentration upon the binding of the CLIP sequence to I-E class II MHC proteins has been identified. Specifically, CLIP was found to be unable to associate with the I-E molecules expressed by the B lymphoma cell lines, A20 (H-2^d), CH27 (H-2^k) or M12.A3 (I-E^d) in both cell-surface and antigen-presentation assays conducted at neutral pH (Figures 5.1–5.4). This ligand also bound with low affinity to purified I-E^d in an enzyme-linked immunoassay at pH 7.0 (Figure 5.5). By contrast, significant binding was observed when the I-E^d molecules and CLIP were incubated together at pH 5.0. These results are consistent with data published in a similar investigation at this time (Bangia & Watts, 1995). These researchers found that biotinylated mCLIP86–102 bound to both purified I-E^d and I-E^k with similar affinity at pH 5.5 (dissociation constants, K_d = 2.3 and 1.1 μM, respectively) but binding was not detectable at pH 7.0.

The effect of pH upon the binding of CLIP to I-E molecules is compatible with earlier reports that high proton concentrations may accelerate the association rates of antigen-derived peptides with class II MHC proteins (*e.g.* Jensen, 1990, 1991; Harding *et al.*, 1991; Wettstein *et al.*, 1991; Mouritsen *et al.*, 1992; Sette *et al.*, 1992b). Indeed, Bangia

& Watts (1995) have determined that CLIP binds optimally to both I-E^d and I-E^k at pH 5.0 which is within the range reported previously for antigenic determinants (pH 4.5–5.5; *e.g.* Jensen, 1990, 1991; Wettstein *et al.*, 1991; Mouritsen *et al.*, 1992; Reay *et al.*, 1992; Sette *et al.*, 1992b). Also of note, these values correspond well to the conditions found within the endosomal environment, where peptide loading occurs *in vivo*.

It has been proposed that acidic conditions promote the protonation of weakly acidic side chains within the class II MHC molecule, which may then provide a favourable environment for the formation of stable complexes (Harding *et al.*, 1991; Jensen, 1991). Recently, this mechanism has been confirmed from the X-ray crystallographic structure of I-E^k complexed with two different antigen-derived peptides (Fremont *et al.*, 1996). At the base of the pocket that accommodates the P6 peptide side chain within the I-E^k peptide-binding groove are situated two acidic residues, αGlu 11 and αAsp 66. Within the local geometry of this pocket, these carboxylic acids face each other and participate in an array of hydrogen bonds with a water molecule and the tightly co-ordinated, neighbouring amino acids, αGln 9, αAsn 62 and αAsn 69. Together with additional hydrogen bonds formed *via* other conserved polar residues, *e.g.* βHis 81, βAsn 82, βTrp 61 and αArg 76, this network contributes positively to peptide binding by making critical contacts with main-chain atoms of the bound peptide to tether it within the I-E groove. Thus, the ability of low pH to facilitate stable peptide binding lies in the capacity of such conditions to protonate both of the acidic residues within the P6 pocket and, thereby, promote the formation of the anchoring hydrogen bond scheme. When a polar amino acid is present within the bound peptide at the P6 position, its side chain may also be involved, as illustrated in the mHb–I-E^k crystal structure, where the P6 residue is glutamic acid (Fremont *et al.*, 1996).

The pH-dependent hydrogen bond network identified in I-E^k involving the αGlu 11 and αAsp 66 residues is likely to be a feature of all I-E variants given the conserved nature of the α-chain sequence (Mengle-Gaw & McDevitt, 1985; Ayane *et al.*, 1986). The involvement of the P6 side chain may also be exhibited by the molecules, I-E^s, I-E^b and I-E^{g7} which share with this allotype the preference for a polar side chain at this position (Schild *et al.*, 1995; Gregori *et al.*, 1999). A similar hydrogen bonding scheme to that in I-E^k may also be predicted for HLA-DR allotypes. These molecules too exhibit conserved acidic residues at positions, α11 and α66. Nevertheless, one report has suggested that the interactions of HLA-DR heterodimers with peptide may still be less sensitive to hydrogen ion concentration than those of I-E (Sette *et al.*, 1992b). Moreover, no such interactions additional to the usual array of main-chain hydrogen bonds have been noted

specifically in any of the HLA-DR X-ray crystallographic analyses to date (Stern *et al.*, 1994; Ghosh *et al.*, 1995; Dessen *et al.*, 1997).

Peptide complexes with I-A class II MHC molecules, in common with those of both HLA-DR and I-E isotypes, also exhibit an elaborate network of hydrogen bonds between conserved polar side chains of the αβ dimer and the main-chain atoms of the peptide backbone, including interactions *via* the MHC residues, αAsn 62 and αAsn 69 (discussed previously in *Chapter 4*, §4.4.2; Fremont *et al.*, 1996, 1998b; Scott *et al.*, 1998). However, I-A^d is the only allelic variant of the I-A isotype to exhibit αGlu 66 (a glycine in I-A^k) while none display an acidic residue at α11. This latter position is occupied by a threonine residue in both I-A^d and I-A^k (Davis *et al.*, 1989). Since the pH-dependent hydrogen bond network has been predicted to require the protonation of two carboxylate groups within the P6 pocket of I-E (Fremont *et al.*, 1996) it is therefore unlikely that an equivalent system is present in I-A molecules. Accordingly, the optimum pH at which I-A^d binds antigen-derived ligands is higher than that for I-E molecules, typically between pH 5.5–6.5 (Jensen, 1991; Sette *et al.*, 1992b). Similarly, the binding of CLIP to I-A^d was not found to be altered dramatically between pH 5.5 and pH 7.0, yielding dissociation constants, K_d, of 2.7 μM and 4.3 μM, respectively (Bangia & Watts, 1995). This finding is consistent with the ability to examine the binding of different CLIP analogues to I-A^d molecules in both antigen presentation and cell-surface binding assays at neutral pH in *Chapters 3* & *4* of this study. Nevertheless, pH-dependent hydrogen bonds may form to some extent between I-A^k αβ dimers and their peptide ligands. For example, these molecules have been reported to make such interactions optimally at more acidic conditions than I-A^d, even so low as pH 4.5 for the HEL46–61 peptide (Harding *et al.*, 1991; Jensen, 1992; Sette *et al.*, 1992b; Mouritsen *et al.*, 1992). Moreover, some features of the P6 pocket in the I-A^k crystal structure are reminiscent of the hydrogen-bonding network at the equivalent site in I-E^k, including a strong preference for a glutamic acid or glutamine at the corresponding position within bound peptide ligands (Fremont *et al.*, 1996, 1998b).

The ability of high proton concentration to enhance the binding of peptides to some class II MHC molecules initially led some researchers to propose that the heterodimer may gain greater conformational flexibility under these conditions, possibly even opening up the structure of the binding groove (Jensen, 1992; Lee *et al.*, 1992). In consideration of the molecular mechanism now believed responsible for these effects (Fremont *et al.*, 1996; Wilson, 1996), it is more likely that any conformational changes within the αβ dimer induced by low pH are very subtle. Consistent with this notion, two-dimensional nuclear magnetic resonance spectra (2D-NMR) of peptide-bound I-E^k molecules have not revealed

any changes in the conformation of the bound peptide as a function of proton concentration, from which it may be inferred that architecture of the binding cleft also does not change (Driscoll *et al.*, 1993). Similarly, far-UV CD measurements have indicated that low pH does not cause any gross alterations with respect to neutral pH in the secondary structure of the class II MHC molecule (Boniface *et al.*, 1996). Instead, by probing the structure of mouse αβ dimers at low pH with the fluorescent dye, ANS (8-anilino-1-naphthalene-sulphonic acid), it has been revealed that these molecules assume a molten globule state associated with increased exposure of internal hydrophobic regions within the pH range where peptide binding is enhanced (Boniface *et al.*, 1996; Runnels *et al.*, 1996). Furthermore, near-UV CD spectra show that protonation appears to perturb the tertiary and/or quaternary interdomain contacts within these molecules to lead to localised unfolding (Boniface *et al.*, 1996). The occurrence of such changes distal to the peptide-binding groove would explain the inability of these effects to be detected in the earlier NMR analysis by Driscoll *et al.* (1993), since that investigation focussed predominantly upon the α-carbon atoms of the bound peptide that are effectively some distance away. Lastly, it is interesting to note from the binding pattern of the ANS dye that these pH-induced structural changes occur in I-E^d, I-E^k and I-A^k at lower pH than for I-A^d (Runnels *et al.*, 1996), consistent with the hypothesis that peptide binding to I-A^k may also be facilitated at low pH.

The actual manner in which the pH-induced structural transitions contribute to enhanced peptide binding in different class II MHC molecules is not clear. Significantly, these changes take place regardless of whether the binding groove is occupied by a high-affinity peptide ligand or not (Boniface *et al.*, 1996; Runnels *et al.*, 1996). Runnels *et al.* (1996) have proposed that some of the hydrogen bonds between peptide main-chain atoms and the residues of the MHC groove may be disrupted at high proton concentration so that only peptides with an adequate source of positive binding energy through side-chain contacts may remain bound. This would serve to increase the pool of class II MHC molecules available for binding peptides. Low pH has indeed been shown to enhance the dissociation of sub-optimally bound peptides (Reay *et al.*, 1992; Sette *et al.*, 1992b). In addition, HLA-DM and HLA-DR molecules show a similar unfolding of hydrophobic domains at low pH which would appear to enhance their intermolecular interactions (Ullrich *et al.*, 1997). Under these conditions, the interaction with HLA-DM is proposed to facilitate peptide loading by stabilising this conformation of HLA-DR. It would be envisaged that a similar situation exists between H-2M and mouse I-E molecules.

The hydrogen bonding interactions in and around the P6 pocket of the I-E peptide-binding groove may serve to explain the general observation that low pH enhances the binding of

peptides to these molecules, however some questions still remain to be answered regarding the binding of CLIP to these molecules. For example, these interactions do not account fully for the inability to detect CLIP–I-E associations at neutral pH — Antigen-derived peptides with similar pH binding optima to that of CLIP still associate with I-E molecules at neutral pH, just less efficiently (Wettstein *et al.*, 1991; Reay *et al.*, 1992; Sette *et al.*, 1992b). Secondly, the pH-dependent hydrogen bond scheme does not provide adequate explanation for the inability to detect naturally-processed CLIP ligands bound to purified I-E heterodimers (discussed previously, §5.1; Rudensky *et al.*, 1991). This phenomenon presumably reflects the dissociation of CLIP from these molecules during isolation of the class II MHC proteins, a procedure which typically involves solubilising the membranes from cell lysates at pH 7.0, followed by affinity chromatography and elution at pH 11.5 (For a description of the methodology used to purify class II MHC molecules, see *Chapter 2*, §2.6). In this sense, it is interesting to consider why mouse CLIP is neither capable of forming nor sustaining a complex with I-E at neutral pH, when human CLIP would appear able to do both with HLA-DR molecules, despite the possible presence of an analogous pH-dependent hydrogen-bonding scheme (Chicz *et al.*, 1992, 1993; Riberdy *et al.*, 1992; Sette *et al.*, 1992a, 1995; Fremont *et al.*, 1996).

The inability of I-E molecules complexed with CLIP to withstand the conditions of neutral pH is particularly unusual given that the P6 hydrogen bond array in the I-E αβ dimer is usually protected well from forces that may disrupt it. For example, it may be envisaged that this network would be threatened by the deprotonation of the α11 and α66 carboxylic acids within this pocket or the entry of solvent into this site to provide alternative hydrogen bonding atoms, however neither of these effects appear to occur. In a general sense, Jensen (1992) has proposed previously that most peptide–class II MHC complexes may remain stable at neutral pH on the cell surface as a result of alterations in the pK_a values of the protonated groups or if the accessibility of these groups to solvent is reduced. Indeed, within the P6 pocket of I-E class II MHC molecules, both of these mechanisms would appear to operate (Fremont *et al.*, 1996). Firstly, the P6 side chain of antigen-derived peptides that bind within the acidic environment of the endosomes *in vivo* is likely to shield the protonated carboxylate groups from the solvent. Secondly, the surrounding protein environment and specific interactions with neighbouring residues would appear to increase the pK_a values of the αGlu 11 and αAsp 66 carboxylate side chains relative to the model value of these groups when free in solution (typically 4.07 and 3.90 at 25°C, respectively; Dawson *et al.*, 1986).

In seeking an explanation for the inability of CLIP to sustain an association with I-E molecules at neutral pH, one may speculate that the rigid form of the Pro 96 residue at P6 in this sequence may not be effective in preventing solvent entry into this pocket when this complex reaches the cell surface. However, it must be noted that CLIP does not dissociate from HLA-DR molecules at the cell surface where Pro 96 is oriented just as unfavourably in this pocket (Ghosh *et al.*, 1995). Indeed, homology modelling of the CLIP–HLA-DR3 interaction has revealed that a hydrogen bond normally found between the amide group of the P6 peptide residue and the conserved αAsn 62 cannot form when proline is accommodated within this site (Lee & McConnell, 1995). In this respect, it is possible that these differences between CLIP binding to I-E and HLA-DR variants may reflect that other side-chain interactions with these latter molecules provide more positive binding energy. In support of this, the replacement of the Pro 96 residue in the CLIP sequence with a polar side chain in the form of lysine (P96K) in this study did lead to stabilisation of CLIP–I-E^k complex at neutral pH in a cell-surface binding assay (Figure 5.10). It is likely that the incorporation of this electropositive component into the P6 pocket would fulfil favourable electrostatic contacts at pH 7.0 when the acidic residues αGlu 11 and αAsp 66 are negatively charged (Table 5.4).

The effect of replacing the P9 residue Met 99 with lysine has also been examined in this study. This substitution created a CLIP ligand that was able to bind to I-E^k molecules under the conditions of a cell-surface binding assay, in fact even more so than P96K CLIP (Figure 5.10). This is presumably due to this residue also fulfilling the particular side-chain preferences of the I-E^k molecules, this time for a basic side chain at the P9 position (Table 5.4; Sette *et al.*, 1989a, 1989c; Marrack *et al.*, 1993; Schild *et al.*, 1995). In this respect, it is interesting to note that Boniface *et al.* (1993) have noted previously the importance of positively-charged residues in natural ligands of I-E molecules and identified a crucial need for such a residue to maintain peptide binding at neutral pH. For example, substitution by L-alanine of the P9 residue of the moth cytochrome *c* peptide, mCytc88–103, Lys 103, increased its dissociation rate at pH 7.0 at least 30 fold. Nevertheless, the substitution of both native residues, Pro 96 and Met 99, by lysine in CLIP did not lead to an additive improvement of binding. It is possible that co-substitution introduces conformational constraints into the peptide such that it is unable to fulfil both molecular bonding requirements at the one time.

In summary, the ability of a given peptide to bind to I-E class II MHC molecules at neutral pH or, similarly, to sustain complex formation under these conditions appears to hinge upon the maintenance of a pH-dependent hydrogen bond network additional to the usual array of these bonds within a peptide–class II MHC complex and/or upon the

presence of suitable anchor residues which provide additional stabilising interactions. At the P6 position, this means either the presence of a polar residue to contribute further to the hydrogen bonding scheme or fulfil electrostatic contacts or, alternatively, a residue of suitable fit to obstruct the entry of solvent into this pocket (Fremont *et al.*, 1996). In consideration of CLIP binding to I-E molecules, the Pro 96 residue is inadequate in all these respects. The maintenance of the I-E complex with a P6-deficient peptide then depends upon there being a sufficient amount of positive binding energy provided through other contact points, such as the critical P9 anchor position (Boniface *et al.*, 1993). Again, the CLIP sequence does not fulfil the preference for a basic residue at this position. Moreover, the CLIP sequence exhibits only one conventional anchor at the P1 position and, instead, appears to stabilise the interaction through anchor residues at the atypical positions, P5, P8 and P12. Collectively, these deficiencies in the CLIP sequence with regards to the I-E peptide-binding motif contribute to the incapacity of this ligand to maintain an effective interaction with these molecules at neutral pH. By comparison, CLIP binds to HLA-DR molecules using a sufficient number of strong anchor side chain contacts *via* some or all of the residues, Met 91, Met 93 and Met 99 (Geluk *et al.*, 1995; Ghosh *et al.*, 1995; Malcherek *et al.*, 1995), such that these complexes are able to persist at neutral pH, even if the hydrogen bonding scheme is weakened by the loss of the carboxylic acid protons.

5.4.5 A self-release mechanism for CLIP binding to I-E molecules?

As discussed in *Chapter 4*, Kropshofer *et al.* (1995a, 1995b) have proposed that the human CLIP sequence possesses features that enable it to facilitate its own release from HLA-DR molecules at low pH independently of the actions of HLA-DM. In this study, the binding of truncated CLIP ligands and CLIP81–104 analogues with single L-alanine substitutions within residues, 81–87, to purified I-E^d molecules has been assessed in competitive immunoassays at pH 5.0 in an effort to determine whether this activity may also be a feature of this ligand binding to I-E $\alpha\beta$ dimers (Tables 5.1, 5.2 & 5.3, Figure 5.9). Although experiments with truncated ligands were not informative, those experiments with the L-alanine-substituted CLIP showed consistently that the replacement of either of the phylogenetically-conserved N-terminal residues, Lys 83 and Pro 87, caused a decrease in the ability of these peptides to bind at pH 5.0. The presence of these residues within the CLIP sequence would thus appears to provide a favourable contribution to the binding interaction with I-E^d under these conditions, whereas the substitution of other residues within the N-terminal region of CLIP had little or no effect on the ability of this ligand to bind to I-E^d.

In a general context, the findings presented herein with the K83A and P87A peptides and I-E^d (Figure 5.9) conflict with the observations of Kropshofer *et al.* (1995a, 1995b) who found that these same N-terminal residues within the wild-type human CLIP sequence contribute to the enhanced dissociation of this ligand from HLA-DR2 and DR3 molecules. However, as with the corresponding experiments with I-A molecules described in *Chapter 4* (§4.4.4), the experiments performed here with I-E^d are difficult to interpret with regard to the CLIP self-release mechanism proposed by Kropshofer *et al.* (1995a, 1995b). For example, the results presented in this study reflect the thermodynamic stability of the complex, whereas those of Kropshofer *et al.* (1995a, 1995b) indicate the kinetic properties of the interaction.

Despite the ambiguous results obtained in this study (Figure 5.9), some intriguing results reported elsewhere do support the notion that certain residues within the N-terminus of CLIP may promote the release of itself or other ligands from I-E heterodimers. Firstly, Brooks *et al.* (1994) have shown that I-E^k heterodimers transfected into the deletion mutant human cell line, T2, are able to form SDS-stable dimers, *i.e.* consistent with human CLIP being able to dissociate from I-E heterodimers independently of the catalytic actions of HLA-DM. Secondly, Adams & Humphreys (1995) have shown that the presence of mouse CLIP77–92 in antigen-presentation assays (termed 'Ii-4' by these investigators) is able to enhance the T cell response to I-E^d- and I-E^k-bound peptides by promoting the exchange of peptides at the cell surface.

5.5. Conclusions

The experiments described in this chapter sought to examine the binding of CLIP to mouse class II MHC molecules of the I-E isotype. In many respects, this interaction has been found to be similar to the manner in which CLIP forms an association with I-A molecules, as determined in *Chapters 3* & *4*. For example, the binding of CLIP to the I-E $\alpha\beta$ dimer occurs through a sequence element of greater than 13-residues in length and which encompasses the phylogenetically-conserved residues, 91–99. Furthermore, binding is supported by a network of hydrogen bonds that forms between the main-chain atoms of the CLIP backbone and conserved residues of the I-E peptide-binding groove.

Unlike the binding of CLIP to I-A molecules, however, the interaction of this ligand with I-E^d has also been shown to involve the formation of a number of strong side-chain anchor contacts, *e.g.* Met 91, Thr 95, Leu 98 and Met 102. The substitution of any of

these residues by L-alanine eliminated the ability of the CLIP sequence to bind. Nevertheless, the presence of anchor contacts is unlikely to preclude degenerate binding of the CLIP ligand to different I-E allelic variants since only one of these interactions is expected to take place within a pocket of the polymorphic peptide-binding groove, that of Met 91 at P1, and residues lining this pocket are relatively conserved. The remaining anchoring side chains are predicted to contact the I-E αβ dimer elsewhere on the molecule where the sequence is also highly conserved. In this respect, promiscuous binding to molecules of the I-E isotype is likely to be facilitated by virtue of the minimal polymorphism in the I-Eα-chain.

A further difference between the interaction of CLIP with I-E molecules and those of the I-A isotype is that this ligand was found to be unable to bind to $I\text{-}E^k$ and $I\text{-}E^d$ at neutral pH but binding did occur at pH 5.0. Antigen-derived ligands have been reported to show a higher affinity for I-E molecules at lower pH as a result of such conditions facilitating the formation of a number of pH-dependent hydrogen bonds in the environment surrounding the P6 pocket within the groove (Fremont *et al.*, 1996). A stable association between the CLIP ligand and $I\text{-}E^k$ was able to be induced at pH 7.0 however, through the provision of an alternative source of positive binding energy in the form of an electrostatically-favourable residue at the P6 and/or P9 position of the peptide sequence.

Thus, in *Chapters 3–5*, the manner in which CLIP interacts with different mouse I-A and I-E class II MHC molecules has been elucidated successfully through an assessment of the binding of a range of different substituted and/or length-altered analogues. Moreover, it has been demonstrated in this chapter that it is possible to modify the CLIP sequence to produce a predictable result using both information on the sequence features of natural ligands, together with details of the spatial arrangement of atoms within the class II MHC peptide-binding groove. Together, these findings serve to illustrate the ability to employ the promiscuous CLIP ligand to probe the peptide-binding properties of a number of different class II MHC variants. In *Chapter 6*, this utility is applied to examine peptide interactions with the atypical class II MHC molecule, $I\text{-}A^{g7}$, from non-obese diabetic (NOD) mice.

CHAPTER 6.

The interaction of CLIP with I-A^{g7}, an atypical class II MHC molecule from non-obese diabetic (NOD) mice

6.1. Introduction

6.1.1 Insulin-dependent diabetes mellitus (IDDM) — an organ-specific autoimmune disease

T cell-mediated immune responses represent a critical component of the vertebrate adaptive immune system and serve to protect the host from potentially life-threatening infections. However, if a T lymphocyte with the propensity to recognise determinants derived from self-proteins escapes the mechanisms that instil immunological tolerance, this process may result in a disease state itself in which the host tissues become the target of immune attack.

One such autoimmune disease in humans is type I or 'insulin-dependent' diabetes mellitus (IDDM). The development of this condition is preceded by a massive infiltration of lymphocytes into the pancreatic islets of Langerhans, a clinically-silent state referred to as insulitis. The actual metabolic disorder that is IDDM arises as the end-stage result of these immune cells mediating the selective destruction of the insulin-producing β cells within the islets. This causes endogenous insulin levels to fall until they are insufficient to maintain glucose homeostasis and affected individuals develop acute hyperglycaemia, a predicament characterised by the accumulation of excessive levels of glucose in the blood stream while the surrounding tissues are starved of an energy source (For a review of IDDM, see Castaño & Eisenbarth, 1990).

As with many other autoimmune diseases, a primary risk factor for susceptibility to IDDM is the inheritance of certain MHC alleles (for reviews on this subject, see Nepom & Erlich, 1991; Campbell & Milner, 1993; Thorsby, 1995; Pugliese & Eisenbarth, 1996). Early investigations revealed a strong risk association between IDDM and the serologically-defined HLA-DR3 and DR4 molecules. Among Caucasian individuals affected by IDDM, 95% express either HLA-DR3 and/or DR4, compared with an occurrence of just 40–50% in unaffected subjects (Wolf *et al.*, 1983). More recently, the development of molecular biology techniques has permitted more precise identification of HLA genetic variants on the short arm of chromosome 6 and has provided an appreciation of those alleles likely to be inherited together through linkage disequilibrium. From these studies, investigators have now refined the primary genetic association of IDDM in Caucasian populations to be at the HLA-DQ locus, which is closely linked to the HLA-DR3 and DR4 haplotypes. The alleles associated with the highest risk are *DQB1*0302* and *DQB1*0201*, expressed in the form of the class II MHC heterodimers, HLA-DQ8

(also known as HLA-DQ3.2; DQA1*0301/DQB1*0302) and HLA-DQ2 (DQA1*0501/DQB1*0201), respectively (Thorsby & Rønningen, 1993). For example, the probability of acquiring IDDM before 15 years of age in Norway is 0.3% (Joner & Søvik, 1989). Of Norwegian IDDM-affected individuals, 81% express HLA-DQ8 molecules, while these are present in only 23% of control subjects (Thorsby, 1995). However the combination of both molecules appears to confer the highest risk: 4% of Norwegian individuals are born DQ2/DQ8 heterozygous, 12% of which will sooner or later develop IDDM (Rønningen, 1997). The closely-linked loci that encode the polypeptide chains of the HLA-DR heterodimer are not without influence and several alleles of the *DRB1*04* type have been identified that accentuate the primary risk of developing IDDM when expressed in association with HLA-DQ8, *e.g. DRB1*0405* (Nepom, 1990; Cucca *et al.*, 1995; Undlien *et al.*, 1997).

The disproportionate over-representation of HLA-DQ8 and DQ2 among IDDM patients, as opposed to control subjects, serves to illustrate the importance of these molecules in acquiring this condition. However, given the incomplete penetrance of this condition, even in individuals expressing both of these particular HLA variants, there are clearly more factors involved. Firstly, there would appear to be a contribution to the disease susceptibility from genetic factors outside of the MHC. This is illustrated by a higher concordance of IDDM development in monozygotic twins (35–50%) than HLA-identical siblings (15–25%; Thorsby & Rønningen, 1993). Indeed, alleles within the HLA complex had been estimated previously to provide only 60–70% of the genetic susceptibility to this condition (Rotter & Landaw, 1984). Another candidate genetic locus that has been examined extensively for its affect on diabetes susceptibility is that encoding the insulin precursor polypeptide, preproinsulin (*e.g.* Bell *et al.*, 1984; Julier *et al.*, 1991; Lucassen *et al.*, 1993; van der Auwera *et al.*, 1993). Secondly, given that the concordance estimates for IDDM in monozygotic twins are not 100%, there must be a strong influence of non-genetic factors in the development of this condition, such as environmental antigenic challenge or geographical location (*e.g.* Ginsberg-Fellner *et al.*, 1984; Bodansky *et al.*, 1986; Helmke *et al.*, 1986). It is interesting to note that factors also exist that protect an individual against IDDM. For example, individuals that express HLA-DQ6 (DQA1*0102/DQB1*0602) develop IDDM rarely, even in the presence of DQ8 or DQ2 (Thorsby & Rønningen, 1993). Similarly, the expression of either of the alleles, *DRB1*0406* or *DRB1*0403*, reduces the risk conferred by the DQ8 molecule (Cucca *et al.*, 1995; Undlien *et al.*, 1997).

6.1.2 The non-obese diabetic (NOD) mouse — an animal model of IDDM

At present, many of the determinants, both inherited and non-genetic, that contribute to susceptibility or resistance to IDDM remain unknown. This makes it extremely difficult to design strategies to treat or, better, to prevent this multifactorial disease. In order to unravel further the aetiology of IDDM, researchers have made extensive use of a number of animal models. These provide a means by which genetic and environmental backgrounds may be manipulated to examine their effect upon the development and progression of autoimmune diabetes.

The most widely studied of the animal models of IDDM is the non-obese diabetic (NOD) mouse. This strain originates from a female mouse that exhibited polyuria (the passage of excessive amounts of urine) and severe glycosuria (the presence of glucose in the urine) accompanied by rapid weight loss, which arose spontaneously during selective inbreeding of outbred Imperial Cancer Research (ICR) mice to derive a cataract-prone strain (CTS; Makino *et al.*, 1980). What makes the NOD mouse such an excellent tool with which to study autoimmune diabetes is the significant number of features of the diabetic disease state that these animals share with human IDDM-affected individuals. Firstly, these two diabetic conditions appear to have a very similar pathology in that, like susceptible humans, NOD mice become diabetic spontaneously following pancreatic autoimmunity, specifically the destruction of the insulin-producing β cells in the islets of Langerhans (Fujita *et al.*, 1982).

Secondly, experiments with MHC congenic mouse strains have shown that the NOD MHC region is critical to the development of diabetes in these animals (Prochazka *et al.*, 1989; Wicker *et al.*, 1992). Indeed, this appears to represent the strongest genetic association for disease susceptibility in these mice (discussed subsequently, §6.1.4). However, on their own, the genes encoded within the complete NOD MHC are still not sufficient to cause diabetes (Hattori *et al.*, 1986; Wicker *et al.*, 1989). Instead, like the human autoimmune diabetic condition, the development of this disease in the NOD mouse is under complex polygenic control (reviewed by Kikutani & Makino, 1992; Baxter & Cooke, 1995; Wicker *et al.*, 1995). Work is currently under way to identify the gene products of a number of the candidate loci which have been localised by linkage analysis with an apparent influence on diabetes penetrance (reviewed by Baxter & Cooke, 1995; Wicker *et al.*, 1995).

Also in common with the human IDDM condition, the incidence of disease in NOD mice appears to be modulated by environmental factors. To date, evidence exists from colonies

of these animals around the world to indicate that both diet and exposure to microorganisms alter the endogenous immunoregulation functions within these mice that affect disease development (Elliott *et al.*, 1988; Oldstone, 1988; Coleman *et al.*, 1990; Leiter *et al.*, 1990; Wilberz *et al.*, 1991). For example, compared with the incidence of disease in clean, pathogen-free NOD colonies, the number of animals contracting this disease is much lower in colonies maintained under conditions where the animals are at risk of microbial contamination (Pozzilli *et al.*, 1993).

It is also of interest to note that, although all NOD mice develop insulitis, the penetrance of the actual diabetic disease state is quite different between female and male mice: a cumulative incidence by 30 weeks of 70–90% in females and 20–40% in males (Makino *et al.*, 1980). Given that castrated males show a higher incidence of diabetes than normal males, while ovariectomised females show a disease incidence more aligned with normal males (Makino *et al.*, 1981), sex-related endocrine factors clearly have an influence upon the development of this disease in NOD mice. In this respect, an examination of the epidemiological figures of human IDDM incidence does not show a similar gender bias, however a higher incidence of disease or greater severity of symptoms in female individuals is observed commonly with other human autoimmune diseases, *e.g.* multiple sclerosis (MS), myasthenia gravis and systemic lupus erythematosus (SLE; Beeson, 1994).

6.1.3 The mechanisms underlying the development of diabetes in NOD mice

An overwhelming amount of experimental data exists regarding the development of autoimmunity and the diabetic condition in NOD mice (*e.g.* see the collection of reviews compiled by Eisenbarth & Lafferty, 1996 and Bach & Mathis, 1997). As with human IDDM, lymphocytic infiltration into the pancreatic islets of NOD mice occurs some time before the observation of overt diabetes. Peri-insulitis in NOD (the presence of lymphocyte infiltrate around the islet in the region containing the pancreatic ducts and blood vessels) may be observed histologically around the time of weaning at 4–5 weeks of age (Fujita *et al.*, 1982). At 6–8 weeks, the islet tissue itself begins to be invaded, leading to the specific destruction of the β cells. Hyperglycaemia is eventually detected in these mice 12–15 weeks after birth and, ultimately, by 30 weeks of age 70–90% of female mice and 20–40% of males exhibit the symptoms of overt diabetes (Makino *et al.*, 1980). An examination of these facts has led some researchers to propose that the disease progression in NOD mice is regulated at two distinct "checkpoints": the first at 3–5

weeks, which involves the initial non-destructive infiltration of lymphocytes into the islets; the second is the later acute change whereupon the β cells are targeted specifically for destruction, culminating in the diabetic disease state (André *et al.*, 1996). The two phases apparent during the course of this disease have also been described as benign and malignant islet-associated autoimmunity, respectively (Gazda *et al.*, 1997; Dilts & Lafferty, 1999).

The elucidation of the factors involved in initiating pancreatic autoimmunity in NOD mice (the initiator phase) and those responsible for the actual destruction of the β cells (the effector phase) has been very difficult. Complicating the situation is the number of different types of inflammatory cells found in the lesions within the pancreatic islets of the diabetic animals, including T cells of both $CD4^+$ and $CD8^+$ lineages, macrophages, dendritic cells and B cells (Kanazawa *et al.*, 1984; Miyazaki *et al.*, 1985; Signore *et al.*, 1989; Jansen *et al.*, 1994).

Regarding firstly the B cells, autoantibodies against islet-cell components are indeed found in the sera of both human individuals predisposed to IDDM and pre-diabetic NOD mice (Reddy *et al.*, 1988; Zimmet *et al.*, 1994; Gianani & Eisenbarth, 1996) — another feature shared between the human and mouse diabetic condition. Analysis of these antibody responses reveals that they are directed frequently against insulin (Palmer *et al.*, 1983; Michel *et al.*, 1989) and the two isoforms of glutamic acid decarboxylase (GAD65 and GAD67), an enzyme which catalyses the synthesis of the inhibitory neurotransmitter γ-aminobutyric acid in neurons and islets cells (GABA; Baekkeskov *et al.*, 1990). However, although at least the GAD-specific humoral reactivity is reported to coincide with the onset of insulitis in NOD mice (Tisch *et al.*, 1993), neither the initiation of anti-islet autoimmunity nor β-cell destruction appear to be antibody-mediated. Indeed, there is little evidence to show that these antibodies themselves are pathogenic (Gianani & Eisenbarth, 1996). Furthermore, NOD mice lacking B cells which still develop both insulitis and diabetes have been reported (Yang *et al.*, 1997), although this is not always the case (Serreze *et al.*, 1996; Akashi *et al.*, 1997; Noorchashm *et al.*, 1997; Wong *et al.*, 1998). Instead, the significance of the B cells within the islet lesions may be as a source of antigen-presenting cells (APC) during the disease process (Noorchashm *et al.*, 1997, 1999; Falcone *et al.*, 1998; King *et al.*, 1998; Serreze *et al.*, 1998; Wong *et al.*, 1998), with the true initiator and effector cells of autoimmune diabetes being T lymphocytes. This has been demonstrated by the ability to induce disease in neonatal or sublethally-irradiated NOD mice by the transfer of splenic T cells from diabetic animals (Wicker *et al.*, 1986; Bendelac *et al.*, 1987). Moreover, neonatal thymectomy reduces the incidence of insulitis and diabetes in NOD mice significantly (Ogawa *et al.*, 1985; Makino *et al.*,

1986). Further experimental work has revealed that CD4$^+$ and CD8$^+$ T lymphocyte subsets are both important to the diabetes disease process but to different extents at distinct stages in its progression (reviewed by Kikutani & Makino, 1992; Wegmann, 1996; Kay *et al.*, 1997; Verdaguer *et al.*, 1997, Wong & Janeway, 1997).

The following is a simple summary of the current thoughts on how the diabetic disease state is believed to arise and the ways in which participant cell types could be involved. Macrophages and dendritic cells (DC) are the earliest cell types to infiltrate the islets at the initiation of the autoimmune process (Jansen *et al.*, 1994). Within this site, these cells have been shown to produce the pro-inflammatory cytokine, tumour necrosis factor-α (TNF-α; Dahlén *et al.*, 1998). Next, the β cells appear to suffer damage, such that an abundance of soluble islet-cell autoantigens is released into the surrounding environment. This may occur directly through the actions of TNF-α, in combination with other cytokines, such as interleukin-1 (IL-1; Baquerizo & Rabinovitch, 1990; Rabinovitch *et al.*, 1990; Delaney *et al.*, 1997). Alternatively, T lymphocyte populations may be recruited to the islets and CD8$^+$ T lymphocytes may inflict acute cytotoxic damage upon the β cells, for example, *via* β-cell class I MHC molecules (Jarpe *et al.*, 1990; Calcinaro *et al.*, 1996; Zechel *et al.*, 1997). Self-antigens released from the damaged β cells are then taken up by APC and presented on their cell surface in association with class II MHC molecules. Limited presentation of these peptides by intraislet APC may also occur without the need for acute lymphocyte-mediated β-cell injury (Shimizu *et al.*, 1995). The APC responsible for presenting β-cell antigens are most likely macrophages, dendritic cells and/or B lymphocytes, since the β cells themselves do not express class II MHC molecules, although they may be induced to do so by particular cytokines (Wright *et al.*, 1986; Pujol-Borrell *et al.*, 1987). Presentation of these antigens is also expected to occur in the draining lymph nodes by migrating APC. In the next critical step, CD4$^+$ T cells with anti-β cell antigen specificities which have escaped the immunological mechanisms for maintaining peripheral tolerance recognise the self-peptides presented by the APC and become activated. The pathogenic CD4$^+$ T lymphocytes are specifically of the T helper 1 (Th1) subset (Rabinovitch, 1994; Katz *et al.*, 1995; Liblau *et al.*, 1995; Pilström *et al.*, 1995), which means that upon their activation, they release further pro-inflammatory cytokines, *e.g.* interferon-γ (IFN-γ). Cytokines of this type are able to provide help to cytotoxic CD8$^+$ T cells to induce clonal expansion and to facilitate macrophage activation, thereby bringing about a considerable expansion of the inflammatory processes. Moreover, the ensuing attack upon the β cells leads to a further increase in the local availability of soluble self-proteins to increase the extent of antigen presentation and T cell recognition. Together, these events have the ultimate result that somewhere amongst this immunological frenzy, the pancreatic β cells are destroyed.

From studies with NOD mice, it has therefore become apparent that the destruction of the β cells occurs as the end result of an inappropriately-directed inflammatory response. Intriguingly, while Th1 cytokines appear to be involved in the progression of the autoimmune process, the administration or induction of cytokines of the opposing Th2 profile (*e.g.* interleukin 4 (IL-4), IL-5, IL-6, IL-10 and IL-13) appears to diminish the intensity of the disease (Rabinovitch, 1994; Liblau *et al.*, 1995; Cohen, 1997b; Elias *et al.*, 1997). This is consistent with Th2 cytokines being important anti-inflammatory agents, with their primary activities lying instead in the promotion of antibody secretion by B cells (for reviews on the Th1/ Th2 paradigm of CD4$^+$ T lymphocyte cytokine production, see Paul & Seder, 1994; Abbas *et al.*, 1996; Romagnani, 1997). Consequently, in very simple terms, it seems likely that the pathogenesis of autoimmune diabetes may be determined ultimately by the overall balance between the response of the Th1/Th2 subsets of CD4$^+$ T lymphocytes.

The exact effector mechanism(s) by which the β cells are destroyed is still unknown, as is the manner by which the other islet cells, such as the glucagon-producing α cells, escape destruction. However, for both of these interrelated events there is no shortage of potential mediators (reviewed by Benoist & Mathis, 1997). For example, CD8$^+$ T cells have been shown to be capable of lysing β cells directly *in vitro* by a perforin-dependent mechanism (Kagi *et al.*, 1996; Utsugi *et al.*, 1996), but it remains unclear as to the exact extent to which this occurs *in vivo*. Macrophages in the islets are also candidates for being the final effectors of β-cell killing (Schwizer *et al.*, 1984), as are both CD8$^+$ and/or CD4$^+$ T lymphocytes by inducing apoptosis through Fas/ Fas ligand interactions (Kurrer *et al.*, 1997). Indeed, Th1 CD4$^+$ cells typically express higher levels of Fas ligand (FasL) than those of the Th2 type (Ramsdell *et al.*, 1994) and inflammatory mediators of the kind present during insulitis have been shown to induce the expression of Fas on β cells (Chervonsky *et al.*, 1997; Stassi *et al.*, 1997). Furthermore, the pancreatic α cells also constitutively express Fas ligand and could contribute themselves to the death of the neighbouring β cells (Signore *et al.*, 1997; for a further discussion of the potential role of Fas–FasL interactions in the destruction of islet β cells, see De Maria & Testi, 1998). Meanwhile, as mentioned above, various soluble factors, such as cytokines and free radical species, have also been shown to induce β-cell death in a non-specific manner (Bendtzen *et al.*, 1986; Nerup *et al.*, 1988; Pukel *et al.*, 1988; Baquerizo & Rabinovitch, 1990; Mandrup-Poulsen *et al.*, 1990; Rabinovitch *et al.*, 1990; Delaney *et al.*, 1997). In this respect, β cells have been reported to be particularly sensitive to damage by oxygen free radicals as a result of their high metabolic rate and the relatively low activity of certain scavenging enzymatic systems within these cells, *e.g.* mitochondrial manganese

superoxide dismutase (MnSOD) and glutathione peroxidase (Malaisse *et al.*, 1982; Asayama *et al.*, 1986).

Also unknown is the identity of the critical diabetogenic determinant towards which T cell tolerance is broken which results in the onset of pancreatic autoimmunity. The discovery of such a molecule would provide an important target for preventive therapies of IDDM, however significant efforts to uncover it have been complicated by the diversity of T cell autoreactivities found within the islet infiltrates of mouse and human diabetic subjects (reviewed by Roep, 1996). In both NOD mice and human IDDM patients, for example, spontaneous T cell responses may be detected against insulin (Naquet *et al.*, 1988; Wegmann *et al.*, 1994) and the 65 kDa GAD isoform, GAD65 (Atkinson *et al.*, 1992; Kaufman *et al.*, 1993; Tisch *et al.*, 1993). T cell reactivity is also observed in NOD mice against both the mouse and human 60 kDa heat shock protein (hsp60) and the derivative peptide, p277 (residues 437–460), antigens which also cross-react with a 65 kDa mycobacterial heat shock protein (Elias *et al.*, 1990, 1991). T cell clones reactive to all of these autoantigens have been derived from the islets of diabetic mice and were shown to secrete Th1 cytokines (IFN-γ and/or TNF-α) and be pathogenic in healthy mouse recipients (Elias *et al.*, 1991; Daniel *et al.*, 1995; Wegmann, 1996; Zekzer *et al.*, 1998).

For some time, such variety in the autoreactive T cell responses in the islets of diabetic individuals has cast doubt on there being a single primary autoantigen responsible for triggering autoimmunity. However further experimentation has revealed that diversification of the immune response may arise simply as the disease progresses; a process known as "determinant spreading" (Steinman, 1995). For example, at 3–4 weeks in NOD mice, concurrent with the onset of insulitis, spontaneous Th1 responses have been found only to GAD of the panel of known autoantigens tested (Kaufman *et al.*, 1993; Tisch *et al.*, 1993). In particular, these responses were directed at a limited region of the GAD protein, although some discrepancy exists over the exact peptide epitope (Kaufman *et al.*, 1993; Zechel *et al.*, 1997, 1998; Chao & McDevitt, 1997). At later time points, the specificity of this T cell autoreactivity was observed to expand intramolecularly to incorporate other determinants within the GAD protein (Kaufman *et al.*, 1993) and, subsequently, the autoreactive T cell repertoire broadened even further, with responses being detected to epitopes on other β-cell antigens, including hsp65 and insulin (Kaufman *et al.*, 1993; Tisch *et al.*, 1993). In this respect, it is interesting to note that the Th1 cytokines that predominate at the site of autoimmune activity may themselves be responsible for inducing the diversification of the epitopes available for presentation — exogenous IFN-γ has been shown to upregulate the expression of cathepsins B, H and L in mouse macrophages, the lysosomal cysteine proteinases critically involved in the

liberation of peptide determinants (Lafuse *et al.*, 1995; Lah *et al.*, 1995). This has been proposed to enhance the stringency of antigen processing to allow the release of previously cryptic epitopes and thereby promote the activation of further autoaggressive T cells of additional specificities (Zechel *et al.*, 1998).

Recently, support for GAD being the primary autoantigen to trigger the onset of pancreatic autoimmunity has been strengthened by the finding that NOD mice may be protected from diabetes by the expression of an antisense GAD gene in the islets, *i.e.* a nucleotide sequence that, when transcribed, complements the mRNA encoding this protein to prevent its translation (Yoon *et al.*, 1999). Analysis of the immunological status of these animals has shown that they exhibit no anti-GAD T cell response, a marked reduction in spontaneous T cell responses to other autoantigens, such as hsp60 and insulin, and T cells from these animals are unable to transfer disease to T cell-deficient NOD recipients. These results are compatible with earlier reports that neonatal or young NOD mice that receive an intrathymic injection of purified recombinant GAD65 or a suspension of islet cells do not develop insulitis or diabetes, presumably through such treatments restoring T cell tolerance to β-cell autoantigens (Tisch *et al.*, 1993; Gerling *et al.*, 1992; Charlton *et al.*, 1994). Nevertheless, some aspects of antisense-GAD NOD mice remain to be assessed, for example, the effect of transplanting normal islets into these animals. Indeed, given that the exact role of GAD in the islets remains unknown, it may be that the downregulation of its expression within this site could simply interfere with the priming of T cells. Thus, the acceptance of GAD as the essential initiating autoantigen must await further experimentation. In this respect, it is interesting to note that the administration of several other known autoantigens, besides GAD, by a number of different routes has also been shown to modulate the severity of the disease symptoms in NOD mice (*e.g.* Zhang *et al.*, 1991; Tisch *et al.*, 1993; 1994; Elliott *et al.*, 1994; Petersen *et al.*, 1994; Elias & Cohen, 1995; Birk *et al.*, 1996; Tian *et al.*, 1996; Bockova *et al.*, 1997; Elias *et al.*, 1997). Several lines of evidence exist to suggest that these self-antigen immunisations protect from disease by inducing a switch from an inflammatory Th1-type response to the production of protective Th2-type cytokines (Tian *et al.*, 1996; Bergerot *et al.*, 1997; Bockova *et al.*, 1997; Cohen, 1997b; Elias *et al.*, 1997; Zechel *et al.*, 1997). It is therefore possible that any significant immunological challenge in these mice may have the potential to halt the progression of the autoimmune cascade. This is compatible with the reduced incidence of diabetes observed in pathogen-exposed NOD colonies (Pozzilli *et al.*, 1993).

Further information on the autoantigens responsible for triggering β-cell autoimmunity might be gained from an analysis of the particular TCR expressed by T lymphocytes

infiltrating the islets of NOD mice. For example, in the rodent model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE), very limited heterogeneity in TCR Vα and Vβ gene usage is observed in the autoreactive T cells, consistent with this disease being triggered by the administration of a single antigen, myelin basic protein (MBP; Acha-Orbea *et al.*, 1988; Zamvil *et al.*, 1988). However, a similar analysis of TCR usage of islet-infiltrating T lymphocytes in 4–5 week old NOD mice reveals a heterogeneous array of TCR gene products (Candéias *et al.*, 1991; Maeda *et al.*, 1991; Nakano *et al.*, 1991). Instead, researchers have had to examine the TCR expression of islet-infiltrating T cells of much younger mice to uncover signs of a monoclonal initiating event in the onset of autoimmune diabetes, *i.e.* T lymphocytes in the islets of 2-week old NOD mice predominantly express a single Vβ8 TCR gene product (Yang *et al.*, 1996). This feature is obscured quickly following the induction of the immune response cascade, consistent with the determinant spreading observed over time in the spontaneous T cell responses to different autoantigens (Kaufman *et al.*, 1993; Tisch *et al.*, 1993). Unfortunately, however, any information regarding the target antigen recognised by these T lymphocytes still awaits the identification of the TCR α-chain(s) expressed by these cells.

Despite considerable advances over the last fifteen years in our understanding of how autoimmune diabetes arises, it is clear that some important pieces of the puzzle are still missing. Further to those mentioned above, given that T cells with anti-self specificities are frequently found in disease-free individuals but show no pathogenic activity towards their target tissues (Schild *et al.*, 1990; Agrawal *et al.*, 1991; Oldstone *et al.*, 1991; Guerder *et al.*, 1994), the mechanism underlying the initial breakdown of peripheral tolerance is unknown. Moreover, it is not understood what triggers the transition from a benign lymphocytic lesion into an aggressive autoimmune assault on the β cells. One theory put forward to explain these issues is that of molecular mimicry (reviewed by Baum *et al.*, 1993). This concept proposes that anergised, self-reactive T cells circulating in the periphery become re-activated following recognition of a high affinity, foreign peptide determinant which, by coincidence, shares sequence or structural homology with the autoantigen. With regards to IDDM, sequence homology does indeed exist between a GAD65 peptide, residues 255–269, and a determinant from the P2-C protein of the Coxsackie B4 virus (residues 32–47; Kaufman *et al.*, 1992), an agent linked previously to the development of autoimmune diabetes (Yoon *et al.*, 1979; D'Alessio, 1992). Furthermore, this same GAD peptide has been shown to elicit T cell responses in approximately 25% of newly-diagnosed IDDM patients tested and similar responses are also generated to the homologous viral determinant (Atkinson *et al.*, 1994).

Nevertheless, more recent studies on the induction of IDDM by the Coxsackie virus now appear to support an alternative theory — that of the so-called 'bystander activation' of autoimmunity (Horwitz *et al.*, 1998). This theory also includes a role for viruses in the development of autoimmune disease but an indirect one, whereby the localised inflammation brought about by a viral infection or superantigen activity creates a disruption of the normal immune processes leading to a breakdown in self tolerance (Benoist & Mathis, 1998; Schiffenbauer *et al.*, 1998). Not mutually exclusive to this, a further theory proposes that a defect may occur in a critical immunoregulatory mechanism, such as the untimely death or anergy of a certain population of CD4$^+$ T cells vital to suppressing autoreactive effector T cells, or a change in their cytokine profile from Th2 to Th1 (*e.g.* Zipris *et al.*, 1991a; Kikutani & Makino, 1992; Shimada *et al.*, 1996; Atkinson, 1997; Bergerot *et al.*, 1997; Lafferty, 1997; Cameron *et al.*, 1998; Salojin *et al.*, 1998). Indeed, the existence of such a population of T cells that counterbalances autoreactive cells is suggested from experimental findings (Boitard *et al.*, 1989; Hutchings & Cooke, 1990; Chosich & Harrison, 1993; Rashba *et al.*, 1993; Singer *et al.*, 1993; Sempe *et al.*, 1994). Alternatively, aberrant selection processes may occur within the thymus during thymocyte development to result in these immunoregulatory T cells existing in insufficient numbers or with inadequate specificities (Thomas-Vaslin *et al.*, 1997).

6.1.4 Do class II MHC molecules play a central role in the development of autoimmune diabetes?

An alternative way to examine the mechanisms by which T cell-mediated immunity triggers the onset of autoimmune diabetes is to investigate specifically how peptides are presented by the particular MHC molecules found within diabetes-stricken individuals, both human and mouse. This seems especially relevant given, firstly, the critical importance of MHC loci in providing susceptibility to this condition as discussed above, secondly, that MHC molecules play a critical role in the development of the T cell repertoire and, thirdly, that immune responses induced by T lymphocytes are ultimately the downstream result of the recognition of peptide fragments presented by MHC molecules.

NOD mice express a unique MHC haplotype, H-2^{g7} (formerly H-2^{nod}). The class I MHC molecules encoded within this set of alleles are serologically-identical to H-2K^d and H-2D^b (Ikegami *et al.*, 1988). In other words, the class I MHC alleles in these mice are no different from those expressed by other inbred mouse strains, such as BALB/c (H-2^d) and

C57BL/6 (H-2^b) mice, *i.e.* mouse strains which do not develop autoimmune diabetes. However, this is not the case with the class II MHC molecules. APC derived from NOD mice exhibit no cell-surface class II MHC heterodimers of the I-E isotype, as a result of a deletion mutation in the promoter region of the gene encoding the Eα-chain (Lund *et al.*, 1990b). This mutation prevents the transcription of this gene, although mRNA is produced for the Eβ-chain (Hattori *et al.*, 1986). Accordingly, I-E cell-surface expression in NOD mice may be restored by backcrossing them with mice transgenic for a functional copy of the I-Eα^d or I-Eα^k gene (Nishimoto *et al.*, 1987; Böhme *et al.*, 1990) or by the direct introduction of an I-Eα^d transgene into fertilised NOD eggs by microinjection (Uehira *et al.*, 1989; Lund *et al.*, 1990a).

Another significant feature of the NOD MHC haplotype is the particular form of H-2A class II MHC molecule expressed in these animals, I-A^{g7}. Sequence analysis of cDNA clones encoding this molecule reveals that the α-chain of the heterodimer is identical to I-Aα^d (Acha-Orbea & McDevitt, 1987). Similarly, the 3' half of the NOD β-chain gene, incorporating the sequence encoding the second external (β_2) domain, the transmembrane and cytoplasmic domains and the 3' untranslated region, is identical to that of the I-Aβ^d gene (for a description of the domain organisation of class II MHC molecules, refer to *Chapter 1*, Figure 1.3). The rest of the NOD I-Aβ^{g7} gene, however, including the sequence encoding the leader peptide, the 5' untranslated region and, most importantly, the sequence of the first external (β_1) domain contains a number of nucleotide differences. On the whole, these differences are not remarkable. In fact, most of the specific nucleotide changes by which the I-A^{g7} β-chain differs from that of I-A^d, are observed in other mouse class II MHC allotypes (Figueroa & Klein, 1986; Acha-Orbea & McDevitt, 1987). Moreover, the estimated 10.8% of nucleotides differing in the β_1 domains between I-A^{g7} and I-A^d conforms with the general level of polymorphism observed in this domain between other Aβ-chain alleles and I-Aβ^d (Acha-Orbea & McDevitt, 1987). However, one region of polymorphism is unique to I-A^{g7} among mouse I-A class II MHC molecules and, indeed, among I-E and human HLA-DR molecules. This is a particular stretch of five consecutive nucleic acid changes between nucleotides, 248 and 252. These substitutions create two codon changes and, in doing so, introduce two radical amino-acid substitutions into the I-A^{g7} β-chain within a region of the polypeptide that is highly conserved within other human and mouse class II MHC molecules: position β56 (Pro → His) and position β57 (Asp → Ser).

The most intriguing aspect of the mutations found in the NOD I-A^{g7} class II MHC molecule is that sequencing of the β-chain of HLA-DQ8 (allele *DQB1*0302*), one of the critical HLA susceptibility molecules of human IDDM patients, reveals a similar loss of

the charged aspartic acid residue at position 57, this time replaced by an alanine (Todd *et al.*, 1987). Furthermore, other class II MHC β-chain alleles that are associated positively with susceptibility to IDDM also lack an aspartic acid residue at this position, having instead neutral residues in the form of serine, alanine or valine, *e.g.* DQB1*0201 of HLA-DQ2 exhibits an alanine at position β57 (Santamaria *et al.*, 1991) and DRB1*0405 expresses βSer 57 (Undlien *et al.*, 1997). By contrast, aspartic acid is found at position 57 in many DQβ allotypes which are neutral or associated negatively with the disease, *e.g.* DQB1*0301 of DQ3.1, DQB1*0602 of DQ6 (Santamaria *et al.*, 1991) and DRB1*0406 (Undlien *et al.*, 1997). A family study examining the identity of the β57 residue as an IDDM protection/susceptibility marker has revealed that, in Caucasian populations, aspartic acid is by far the most common residue found at this position in non-diabetic individuals but it is significantly less frequent in patients with IDDM (Morel *et al.*, 1988). Together, these findings suggest that the amino acid at position 57 in the β-chain of class II MHC molecules is a significant determinant in influencing susceptibility to autoimmune diabetes.

The importance of residue 57 in the class II MHC β-chain in predisposing individuals to autoimmune diabetes has been confirmed by the finding that the incidence of spontaneous diabetes is reduced in transgenic NOD mice that exhibit heterozygous expression of other I-A class II MHC molecules that exhibit βAsp 57 (Miyazaki *et al.*, 1990; Slattery *et al.*, 1990; Singer *et al.*, 1993), or express the Aβ^{g7} polypeptide where residue Ser 57 has been altered by site-directed mutagenesis to Asp (Quartey-Papafio *et al.*, 1995). Nevertheless, in most cases, these transgenic NOD mice still feature some degree of insulitis (Slattery *et al.*, 1990; Singer *et al.*, 1993; Quartey-Papafio *et al.*, 1995). Moreover, NOD mice expressing Aα^{k}Aβ^{k} in which position β57 has been mutated from aspartic acid to serine are also protected against developing overt diabetes (Miyazaki *et al.*, 1990). Thus, despite the importance of this residue in contributing to diabetes development, residue β57 alone is not sufficient to induce β-cell autoimmunity, a finding consistent with the known multifactorial nature of IDDM. Indeed, in some instances the so-called "codon 57" model of diabetes susceptibility does not appear to be applicable at all. For example, Caucasian individuals that are homozygous at the *DQB1* locus for Asp 57-positive alleles may still develop IDDM, while individuals expressing other *DQB1* genotypes that lack aspartic acid at this position never become diabetic (Rønningen *et al.*, 1989; Baisch *et al.*, 1990). Furthermore, the *DQB1* alleles found with increased frequency in Japanese IDDM patients do exhibit an aspartic acid residue encoded at position β57 (Awata *et al.*, 1990; Ikegami *et al.*, 1990a). In the Bio-Breeding (BB) rat model of autoimmune diabetes, these rats, like NOD mice, express an class II MHC

molecule that lacks aspartic acid at position 57 (RT1.B^{u}), however so too does the Lewis rat which is not susceptible to this condition (RT1.B^{l}; Chao *et al.*, 1989).

It is of interest to note that the expression in NOD mice of a transgene encoding a modified Aβ^{g7} chain exhibiting a His → Pro mutation at position β56, *i.e.* a reversion to the conserved mouse wild-type residue, also protects these animals from diabetes but, moreover, reduces the severity of insulitis as well (Lund *et al.*, 1990a). Similarly, double transgenic NOD mice expressing the I-A^{g7} β-chain gene in which the codons for both βHis 56 and βSer 57 have been mutated to encode a proline and aspartic acid respectively — the so-called NOD.PD mice — develop neither substantial insulitis nor overt diabetes (Singer *et al.*, 1998). Thus, both β-chain residues, 56 and 57, of the I-A^{g7} NOD class II MHC molecule exhibit the potential to influence profoundly the diabetogenic nature of the entire αβ dimer, albeit to different extents. The mutation at β56 appears unlikely, however, to be a contributing factor in human IDDM since a proline residue at the β56 position is conserved throughout HLA-DQ molecules (Todd *et al.*, 1987). Also noteworthy, the effect of these I-A^{g7} β-chain residues, 56 and 57, upon susceptibility to autoimmune diabetes in NOD mice appears to be secondary to the presence of a functional I-E class II MHC molecule — the expression of an I-Eα transgene in these animals prevents overt autoimmune diabetes and reduces insulitis (Nishimoto *et al.*, 1987; Uehira *et al.*, 1989; Böhme *et al.*, 1990; Lund *et al.*, 1990a).

6.1.5 The mutations at positions β56 and β57 in I-A^{g7} may affect the binding of peptides

How then do the β56 and β57 residues of I-A^{g7} exert their influence upon the development of β-cell autoimmunity in NOD mice? Examination of X-ray crystallographic images of different human and mouse class II MHC αβ dimers (Brown *et al.*, 1993; Stern *et al.*, 1994; Ghosh *et al.*, 1995; Fremont *et al.*, 1996, 1998b; Scott *et al.*, 1998), together with an homology model of I-A^{g7} (Reizis *et al.*, 1997b), locates the residues 56 and 57 within the β-chain H1 α-helix that forms part of one of the walls of the peptide-binding groove, *i.e.* in close proximity to the C-terminal region of the bound peptide ligand (for a description of the structure of the class II MHC peptide-binding groove, refer to *Chapter 1*, Figure 1.4). In particular, the aspartic acid residue typically found at position 57 of the wild-type class II MHC β-chain is critically involved in stabilising this distal region of the peptide-binding groove. This is by consequence of the aspartyl carboxylate group participating in a salt bridge with the guanidino group of an arginine residue at position 76 in the α-chain directly beneath the exiting peptide, thereby

providing a vital link between the α_1 and β_1 helical domains (Brown *et al.*, 1993; Stern *et al.*, 1994; as stated previously, class II MHC residues are numbered throughout this thesis according to the secondary structure-based sequence alignment of the α_1 and β_1 domains of the molecules, I-A^k, I-E^k and HLA-DR, reported by Fremont *et al.*, 1998b). The inability of this electrostatic interaction to form in I-A^{g7} due to the Asp → Ser mutation at the β57 position may therefore result in these molecules being inherently unstable. This could contribute to the development of autoimmunity in NOD mice by altering the capacity of these molecules to present peptides efficiently to CD4$^+$ T lymphocytes for both thymic selection and/or for subsequent peripheral immune responses.

Also influencing the ability of class II MHC molecules to present peptides effectively, both the βAsp 57 and αArg 76 residues that are involved in the class II MHC salt-bridge have the potential to form hydrogen bonds, either directly or indirectly *via* water molecules, with amide hydrogens atoms and carbonyl oxygen atoms of the peptide backbone (Stern *et al.*, 1994; Ghosh *et al.*, 1995; Fremont *et al.*, 1996, 1998b; Weber *et al.*, 1998). This situation is equivalent to that seen in class I MHC molecules where the side chains of the conserved residues, Tyr 84 (α_1 domain) and Thr 143 (α_2 domain), occupy the positions analogous to αArg 76 and βAsp 57, respectively (for a description of the domain organisation of class I MHC molecules, refer to *Chapter 1*, Figure 1.3; Brown *et al.*, 1988; Madden *et al.*, 1991; Fremont *et al.*, 1992; Madden *et al.*, 1992; Silver *et al.*, 1992). Together, the Thr 143 and Tyr 84 in the class I MHC three-dimensional structure are positioned to hydrogen bond with the oxygen atoms of the peptide C-terminus carboxylate group, thereby tethering this end of the peptide in place. In the class II MHC heterodimer, electrostatic restraint of this region of the bound peptide is less important, given the continuation of the peptide beyond this site, the absence of a C-terminal carboxylate and the number of other hydrogen bonds occurring elsewhere along the length of the peptide. Nevertheless, a hydrogen bond formed between the βAsp 57 residue and peptide main-chain atoms could provide a vital contribution to the overall stability of a peptide–class II MHC complex in terms of whether it is sufficiently long-lived to be recognised by CD4$^+$ T lymphocytes at the cell surface. In this way, the possibility that the serine residue at position β57 does not form such a bond in peptide-bound I-A^{g7} class II MHC complexes, or that it is of lesser strength, may well influence the efficacy of the deletion of self-reactive T cell clones in the thymus and/or their subsequent activation in the periphery.

Another possible contribution of the β57 mutation in I-A^{g7} to the development of autoimmunity may result from an alteration in the peptide repertoire presented by these

class II MHC molecules. The location of residue β57 at the base of the peptide-binding groove coincides with the pocket that accommodates the side chain of the P9 residue of the bound peptide. Thus, the βAsp57Ser mutation in I-A^{g7} may influence the side-chain specificity of this particular binding site. For instance, in other mouse class II MHC molecules of the I-A isotype in which the β57–α76 salt bridge is intact, the P9 pocket is small but relatively degenerate between allotypes, tolerating a range of peptide side chains of both aliphatic and polar characteristics (discussed in *Chapter 3*, §3.4.4 & Table 3.2). However, the inability to form the salt bridge at this site in I-A^{g7} leaves the αArg 76 amino acid unpaired. This drives a preference for I-A^{g7} to bind peptides that are able to make a compensatory interaction with the αArg 76 positive charge, the fulfilment of which should provide considerable positive binding energy to the interaction. Accordingly, sequencing of naturally-processed peptide ligands bound to I-A^{g7} has revealed that these peptides frequently exhibit negatively-charged amino acid residues at the P9 position in their C-terminus (Reich *et al.*, 1994).

Regarding the capacity of the Pro → His mutation at position β56 to affect the development of diabetes, X-ray crystallographic structures of mouse and human class II MHC molecules reveal that this residue is chiefly oriented away from the peptide-binding groove (*e.g.* Brown *et al.*, 1993; Stern *et al.*, 1994; Fremont *et al.*, 1998b; Scott *et al.*, 1998). However, it is likely that the βPro56His mutation may too affect antigen presentation by the I-A^{g7} molecule *via* effects on both the stability and specificity of peptide binding. For example, the substitution of the rigid cyclic proline residue for the more flexible histidine in I-A^{g7} will undoubtedly introduce changes into the conformation of the H1 α-helix of the β-chain at this site (*Chapter 1*, Figure 1.4) which then may impact significantly upon the stability of the αβ complex and/or its ability to form the typical hydrogen-bond network and side-chain interactions with peptide ligands. Moreover, this mutation introduces a positive charge into this position which may influence further the preference for negatively-charged residues at the C-terminus of I-A^{g7}-binding peptides, as described above for the βAsp 57 mutation.

Thus, the location of both the β56 and β57 residues within the environment of the class II MHC peptide-binding groove makes it is possible to envisage that amino acid changes at these positions within the I-A^{g7} heterodimer may impose significant changes upon the ability to bind peptides and the specific sequence features of the peptides that they do bind. Such effects would indeed have ramifications on the presentation of antigen to CD4$^+$ T lymphocytes during both thymic selection and later in the periphery, which may well be of such significance as to trigger autoimmunity. To examine this hypothesis, an analysis of how peptides bind to the wild-type I-A^{g7} molecule and the manner by which

the NOD-specific β-chain mutations in the peptide-binding groove influence ligand binding is clearly needed.

Presently, the I-A^{g7} class II MHC αβ dimer is believed to be intrinsically unable to form strong interactions with peptide ligands. However, these conclusions have been drawn predominantly from the behaviour of these molecules when subjected to SDS-PAGE. Specifically, it has been reported that only a small percentage of these molecules is able to withstand dissociation of the αβ subunits in the presence of SDS detergent (Carrasco-Marin *et al.*, 1996; Harrison *et al.*, 1997; Reizis *et al.*, 1997b; Nabavieh *et al.*, 1998). As discussed in *Chapter 3* (§3.4.6), this is a rather arbitrary approach for determining the overall complex stability and, thereby, assessing the efficiency of peptide binding. Firstly, the molecular basis of SDS-stability is only now becoming understood (*e.g.* Nelson *et al.*, 1996; Natarajan *et al.*, 1999b) while the functional consequences of this feature *in vivo*, if any, are still unknown. In this respect, even the majority of I-A^{d} class II MHC molecules are found to be unstable in the presence of SDS (Carrasco-Marin *et al.*, 1996). Secondly, peptides different from the range of natural ligands accessible *in vivo* could possibly be acquired during cell lysis and protein purification, or simply as a result of using different growth medium preparations. These may yield a false representation of the stability state of the αβ dimer under examination. Indeed, even within the different SDS-PAGE analyses of the I-A^{g7} molecule, significant variation in the degree of susceptibility to dissociation has been found. For example, Harrison *et al.* (1997) reported that greater than 95% of I-A^{g7} molecules affinity-purified from detergent lysates of a B cell hybridoma dissociate into the component monomers when subjected to SDS-PAGE. Similarly, Carrasco-Marin *et al.* (1996) have reported that just 1% of radiolabelled I-A^{g7} molecules that are immunoprecipitated from NOD mouse splenic cells migrate as stable αβ dimers when subjected to SDS-PAGE in sample buffer containing 2% SDS. This is in contrast to the 12% of compact I-A^{d} molecules, 39% of I-A^{k} and 78% of I-A^{b} detected in the same study. Similar results were also obtained when the class II MHC molecules were detected by Western blotting using an antibody against the cytoplasmic portion of the α-chain. However, Reizis *et al.* (1997b) have found that up to 25% of metabolically-labelled and cell-surface-labelled I-A^{g7} immunoprecipitated from NOD mouse splenocytes were in a compact conformation and resistant to SDS-induced dissociation.

A more reliable approach is therefore required to determine fully the biochemical characteristics of peptide ligand interactions with I-A^{g7}. In this respect, the extensive array of different substituted and/or length-altered analogues of the promiscuous class II MHC-binding Ii peptide, CLIP, available in this laboratory provides an excellent resource

with which the ability of a single given peptide to bind to I-A^{g7} may be investigated in some detail. The information produced in this manner on the specific molecular contacts formed between ligand and class II MHC molecule may provide a good indication as to the extent to which the binding of the peptide yields energy to stabilise the complex. The success of such analyses has been proven previously in *Chapters 3–5* through the elucidation of the molecular basis by which the CLIP ligand interacts with mouse class II MHC molecules of both I-A and I-E isotypes.

The investigation into the effects of the mutations at positions β56 and β57 in the I-A^{g7} class II MHC molecule on CLIP binding may be extended further by comparing the results obtained with wild-type I-A^{g7} with those of single amino acid revertants. In this respect, Quartey-Papafio *et al.* (1995) have generated previously three transfectant B cell lines, M12.NOD, M12.PRO and M12.ASP, from the I-A^{-} cell line, M12.A3 (Glimcher *et al.*, 1985), all of which have been provided to this laboratory as part of a collaborative study. The M12.NOD cell line expresses wild-type I-A^{g7} molecules, while the M12.PRO and M12.ASP cell lines express variants of the I-A^{g7}, each with a single amino acid mutation, βHis56Pro and βSer57Asp, respectively. In other words, these latter two cell lines express at the respective β56 and β57 positions the residue conserved in other mouse class II MHC molecules.

In this study, the ability of a broad array of substituted and/or length-altered CLIP analogues to bind to wild-type I-A^{g7} and the single amino acid-substituted variants I-A^{g7Pro56} and I-A^{g7Asp57} has been assessed in cell-surface peptide binding assays. This has been achieved using the transfected mouse B lymphoblastoid cell lines, M12.NOD, M12.PRO and M12.ASP, respectively, as constructed by Quartey-Papafio *et al.* (1995). Data yielded on the stability and peptide-binding behaviour of the wild-type NOD class II MHC αβ dimer, I-A^{g7}, have been compared and consolidated with previous biochemical analyses of this molecule and the particular effects on peptide binding of the βHis56Pro and βSer57Asp mutations within the I-A^{g7} molecule have been evaluated. Comparisons of these findings have also been made with results obtained from other mouse class II MHC molecules, such as those examined previously in *Chapters 3–5*. Given the critical role of antigen presentation by class II MHC molecules in shaping the CD4^{+} T cell repertoire (*Chapter 1*, §1.7.2) and in mediating cellular immune responses (*Chapter 1*, §1.7.1), possible mechanisms by which I-A^{g7} may contribute to the development of autoimmune diabetes in NOD mice are discussed.

6.2. Materials and Methods

All experimental procedures performed in this chapter are detailed in *Chapter 2*. Briefly, the mouse B cell lines, M12.A3 (I-E^d), M12.NOD (I-E^d, I-A^{g7}), M12.PRO (I-E^d, I-A$^{g7\beta Pro\ 56}$) and M12.ASP (I-E^d, I-A$^{g7\beta Asp\ 57}$) were provided generously by Dr A. Cooke, University of Cambridge, UK, as part of a collaborative study into the effects on ligand interactions of the βHis 56 and βSer 57 residues within the peptide-binding groove of the I-A^{g7} class II MHC molecule. The I-A$^-$ parental cell line, M12.A3, was derived from the H-2^d B cell lymphoma, M12.4.1, by γ-irradiation mutagenesis followed by negative selection with anti-I-A^d mAb (Glimcher *et al.*, 1985). The transfected cell lines, M12.NOD, M12.PRO and M12.ASP were generated by Quartey-Papafio *et al.* (1995) by the transfection of Aβ DNA constructs into M12.A3 cells. The mouse B cell lines, A20 (H-2^d), CH27 (H-2^k), M12.D (I-A^d) and 1-5.4 (I-A^u), were also used as APC in cell-surface binding assays. All of these cell lines are detailed in *Chapter 2*, Table 2.1.

The L-alanine- and D-alanine-substituted CLIP86–104 analogues used in the competitive binding assays have been listed previously in *Chapter 3*, Table 3.1 and *Chapter 4*, Table 4.1, respectively. Truncated and frameshifted CLIP analogues are listed within §6.3.4 in Tables 6.1 and 6.2, respectively, with further substituted and/or length-altered CLIP ligands described in Table 6.3. The set of CLIP86–104 analogues exhibiting amino acid substitutions at positions 99 and/or 100 are shown in Table 6.4 within §6.4.5. Sequences of the I-A^{g7}-binding antigen-derived peptides, MSA560–574 and p12(166–185), are given in Figure 6.7a and b, respectively. Sequences of the antigen-derived peptides, rMOG8–22 and HEL10–23, are given in Figure 6.8a and b, respectively. Peptide concentrations used in binding assays are stated in the figure legends. In all competitive cell-surface binding assays (*Chapter 2*, §2.7.2), competitor peptides were tested over the concentration range, 16–250 μM.

I-A^{g7} molecules were purified from a detergent lysate of the transfected cell line, M12.NOD, by affinity chromatography on a 10-3.6.2 mAb column using the standard protocol outlined in *Chapter 2*, §2.6. Other affinity-purified mouse class II MHC molecules shown in Figure 6.1 were prepared similarly on mAb columns as follows: I-A^k; 10-3.6.2, I-A^d; MK-D6, I-E^d & I-E^k; 14.4.4S. Tris-tricine SDS-PAGE and Western blotting of purified class II MHC molecules were performed as described in *Chapter 2*, §2.5.2 and §2.5.4, respectively, with one amendment: I-A^{g7} and I-A^k molecules were detected by immunoblotting with the unconjugated primary mAb, 10-2.16, followed by horseradish peroxidase-conjugated goat anti-mouse IgG (GαM–HRP). I-A^d and I-E^{d,k}

were detected with biotinylated MK-D6 and 14.4.4S, respectively, followed by horseradish peroxidase-conjugated streptavidin (S–HRP).

6.3. Results

6.3.1 SDS-PAGE is not a reliable technique with which to determine the peptide-binding capacity of class II MHC molecules

I-A^{g7} class II MHC molecules have been reported to be largely unstable in the presence of the ionic detergent, SDS and have therefore been deemed to bind peptides poorly (Carrasco-Marin *et al.*, 1996, 1997; Harrison *et al.*, 1997; Reizis *et al.*, 1997b; Nabavieh *et al.*, 1998). However, SDS-PAGE experiments are frequently subject to considerable variation. To illustrate this point, shown in Figure 6.1 is an example of one such experiment performed in this laboratory with affinity-purified I-A^{g7}. SDS-PAGE was conducted on a 12–15% Tris-tricine gradient gel followed by immunoblotting and detection with the anti-I-A$\beta^{k,u,g7}$ mAb, 10-2.16 (Oi *et al.*, 1978). The strong band at approximately 60 kDa indicates that most of these molecules are in a compact conformation and thereby resistant to SDS-induced dissociation of their constituent subunits. Little monomeric β-chain is visible at approximately 30 kDa. Some degradation of the 10-3.6.2 antibody in the affinity column used for the I-A^{g7} purification seems to have occurred, as apparent from the detection of immunoglobulin heavy and light chains (approximately 53 kDa and 23 kDa, respectively) in the I-A^{g7} sample blotted with both 10-2.16 and horseradish peroxidase-conjugated goat anti-mouse IgG (GαM-HRP; lane 1) and the background control sample of I-A^{g7} blotted with GαM-HRP only (lane 2). Such degradation of the purifying mAb was not observed in the I-A^{k} preparation purified from a CH27 (H-2^{k}) detergent lysate on a similar 10-3.6.2 column when blotted with these same reagents (lane 3). This and other mouse class II MHC molecules affinity-purified in this laboratory are shown alongside the I-A^{g7} samples for comparison of the degree of stability observed typically with each of these molecules in SDS: I-A^{d} (lane 4), I-E^{d} (lane 5) and I-E^{k} (lane 6).

In other words, affinity-purification of I-A^{g7} in this laboratory appeared to yield a high proportion of αβ dimers resistant to SDS-induced dissociation, even in the presence of SDS at a final concentration of 4%. (Such assays are most frequently performed in sample buffer with a final concentration of 0.2–2% SDS. For example, see the original SDS stability protocols of Billing *et al.*, 1976; Springer *et al.*, 1977; Pious *et al.*, 1985).

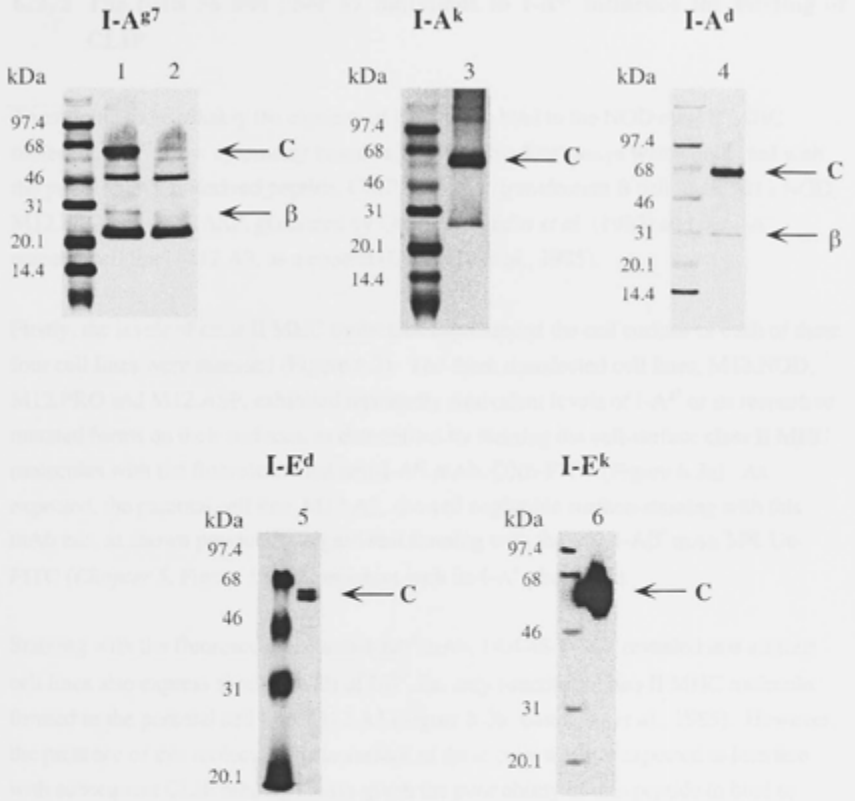


Figure 6.1 *I-A^{g7} produces compact dimers upon SDS-PAGE*

Mouse class II MHC proteins were purified from detergent lysates of B cell lines by immunoaffinity chromatography, as described in *Chapter 2* (§2.6): I-A^{g7}, M12.NOD, I-A^{k} & I-E^{k}, CH27; I-A^{d} & I-E^{d}, A20. Pooled fractions containing purified class II MHC proteins were separated by SDS-PAGE on either a 10–20% tris-tricine gradient gel (I-A^{g7}, I-A^{k}, I-A^{d}) or a 12% tris-glycine gel (I-E^{d}, I-E^{k}) and transferred to nitrocellulose membranes for immunodetection (*Chapter 2*, §2.5): I-A^{g7} & I-A^{k}, 10-2.16 + GαM-HRP; I-A^{d}, biotinylated MK-D6; I-E^{d} & I-E^{k}, biotinylated 14.4.4S. Compact class II MHC αβ dimers are indicated by arrow C. Monomeric β-chain is indicated by arrow β.

6.3.2 The βHis 56 and βSer 57 mutations in I-A^{g7} influence the binding of CLIP

To examine more reliably the capacity of peptides to bind to the NOD class II MHC molecule, I-A^{g7}, flow cytometry-based cell-surface binding assays were conducted with the promiscuous Ii-derived peptide, CLIP, using the transfectant B cell lines, M12.NOD, M12.PRO and M12.ASP, generated by Quartey-Papafio *et al.* (1995) and the I-A^{-} parental cell line, M12.A3, as a control (Glimcher *et al.*, 1985).

Firstly, the levels of class II MHC molecules expressed at the cell surface of each of these four cell lines were assessed (Figure 6.2). The three transfected cell lines, M12.NOD, M12.PRO and M12.ASP, exhibited repeatedly equivalent levels of I-A^{g7} or its respective mutated forms on their surfaces, as determined by staining the cell-surface class II MHC molecules with the fluoresceinated anti-I-A^{g7} mAb, OX6-FITC (Figure 6.2a). As expected, the parental cell line, M12.A3, showed negligible surface-staining with this mAb nor, as shown previously, significant staining with the anti-I-Aβ^{d} mAb MK-D6-FITC (*Chapter 5*, Figure 5.4a), consistent with its I-A^{-} phenotype.

Staining with the fluoresceinated anti-I-Eα^{d} mAb, 14.4.4S-FITC, revealed that all four cell lines also express similar levels of I-E^{d}, the only functional class II MHC molecule formed in the parental cell line, M12.A3 (Figure 6.2b; Glimcher *et al.*, 1985). However, the presence of this molecule on the surface of these cells was not expected to interfere with subsequent CLIP binding assays given the poor ability of this peptide to bind to molecules of the I-E isotype under such conditions, as discussed in *Chapter 5* (§5.4.4). Subsequently, this assumption was proven to be correct (see below).

Next, the direct binding of biotinylated CLIP86–104 to the four cell lines, M12.A3, M12.NOD, M12.PRO and M12.ASP, was assessed. As shown in Figure 6.3, concentration-dependent binding of this peptide to surface class II MHC molecules was readily detectable by flow cytometry for the three cell lines expressing forms of the I-A^{g7} αβ dimer (filled symbols). By contrast, very little binding of the CLIP ligand was observed to the M12.A3 cell line which expresses only I-E^{d} at the cell surface (open squares). Consistent with the data presented previously (*Chapter 5*, Figure 5.4), it may thus be assumed that the fluorescence signal generated with the cell lines M12.NOD, M12.PRO and M12.ASP is the result of the biotinylated CLIP86–104 peptide binding only to the particular forms of cell-surface I-A^{g7} molecules expressed by these cells.

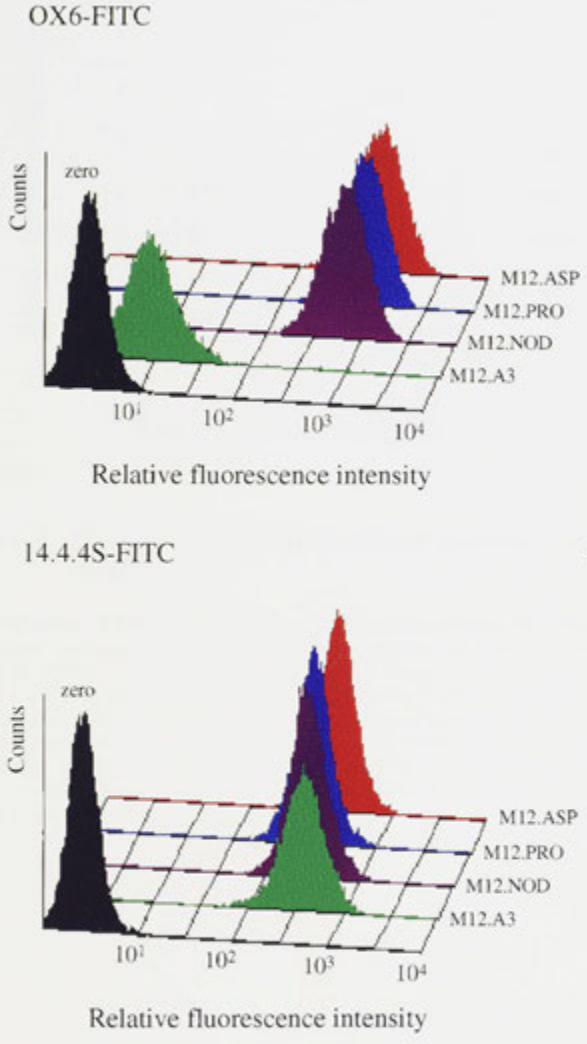


Figure 6.2 *Expression of I-A^{g7} and I-E^{d} class II MHC molecules on the surface of transfected mouse B lymphoblastoid cell lines.*

The three transfected cell lines, M12.NOD (I-A^{g7}, I-E^{d}), M12.PRO (I-A^{g7Pro56}, I-E^{d}) and M12.ASP (I-A^{g7Asp57}, I-E^{d}), together with the parental cell line, M12.A3 (I-E^{d}), were stained with the fluoresceinated mAb **(a)** OX-6 (recognising I-A^{f,k,r,s,u,g7}) or **(b)** 14.4.4S (recognising I-E^{d,k,p,r,u}) to determine their relative levels of expression of class II MHC molecules. A control sample of M12.NOD cells was stained with avidin-FITC to depict zero fluorescence. Flow-cytometric analysis was conducted as described in *Chapter 2*, §2.7.2.

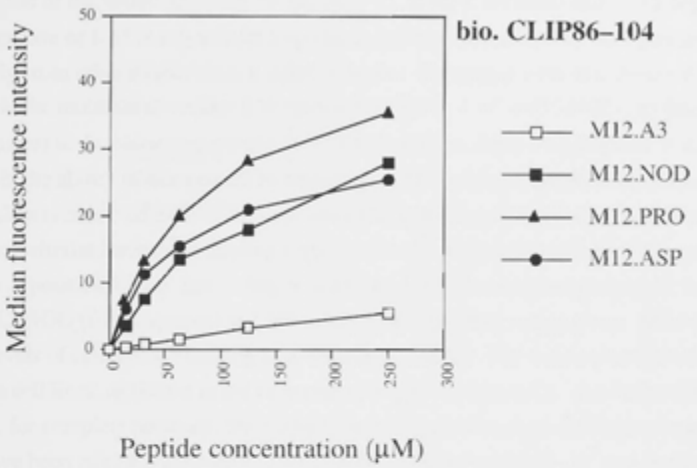


Figure 6.3 *The binding of CLIP86–104 to mutated I-A^{g7} class II MHC molecules.*

Biotinylated mouse CLIP86–104 was examined for its ability to bind to the mutated forms of the I-A^{g7} class II MHC molecule expressed by the M12.A3-derived cell lines, M12.PRO (I-A^{g7Pro56}) and M12.ASP (I-A^{g7Asp57}). Binding was compared to that obtained with M12.NOD cells, which express wild-type I-A^{g7} and, with the parental cell line, M12.A3. Data shown are representative of seven independent assays, all yielding equivalent results. Binding is expressed as median fluorescence intensity (MFI) from which background signals, measured in the absence of biotinylated peptide, have been subtracted (M12.A3, 3.40; M12.NOD, 2.69; M12.PRO, 3.05; M12.ASP, 2.64). MFI measurements have also been normalised for any minor differences in the levels of class II MHC expression between these cell lines, as determined by staining with OX6-FITC. Cells were incubated with biotinylated CLIP for 18 hours at 37°C before flow cytometric analysis, as described in *Chapter 2*, §2.7.2.

The location of the mutations, βHis 56 and βSer 57, at the C-terminal end of the peptide-binding groove of I-A^{g7} has led to the hypothesis that this molecule may bind peptides differently from other mouse class II MHC variants. Consistent with this, Figure 6.3 shows that the mutation of residue β56 from a histidine in I-A^{g7} (M12.NOD cell line; filled squares) to the conserved proline (M12.PRO cell line; filled triangles) led to an increase in the ability of this peptide to bind to these cell-surface class II MHC molecules. This result was observed each of the seven times that this experiment was performed. However, a similar increase in binding capacity was not recorded for the similar reversion mutation at position 57, *i.e.* Ser → Asp between the I-A^{g7} molecules expressed by the cell lines, M12.NOD (filled squares) and M12.ASP (filled circles), respectively. Most often, similar levels of cell-surface binding of biotinylated CLIP86–104 were observed between these two cell lines, as shown in the representative plot in Figure 6.3. It is important to note that, for complete accuracy, the median fluorescence intensities (MFI) shown in this figure have been adjusted for even slight differences in the levels of I-A^{g7} expression observed with OX6-FITC staining between these cell lines on the day of experiment. This normalisation has been performed throughout this chapter where peptide binding levels between the different cell lines have been compared directly.

Another way in which to investigate the ability of a peptide to bind to cell-surface class II MHC molecules is to establish competition between the given labelled peptide and its equivalent unlabelled form. This provides very specific information regarding peptide-binding capacity at only those sites where labelled and unlabelled ligand are in direct competition. To examine further the effects of the β56 and β57 mutations on peptide binding to I-A^{g7}, competitive cell-surface binding assays were conducted, measuring the binding of biotinylated CLIP86–104 in the presence of unlabelled analogue (Figure 6.4). From the results of the previous direct titration of biotinylated CLIP in Figure 6.3, a concentration of 50 μM of this peptide was chosen for these assays with each of the cell lines, M12.NOD, M12.PRO and M12.ASP.

Unbiotinylated CLIP86–104 inhibited the signal generated by the binding of the biotinylated CLIP86–104 to the M12.NOD, M12.PRO, and M12.ASP cells in a concentration-dependent manner, consistent with these two peptides competing for the same single class of binding site (Figure 6.4). Interestingly, unlabelled CLIP86–104 (filled squares) did not compete as well with the biotinylated form for binding to the wild-type I-A^{g7} molecules expressed on the M12.NOD cell surface (Figure 6.4a) as it did to the βHis56Pro mutated form of I-A^{g7} expressed by the M12.PRO cells (I-A^{g7Pro56}; Figure 6.4b). For example, the concentration of unbiotinylated CLIP86–104 required to inhibit 50% of the biotinylated CLIP signal with M12.NOD cells (IC_{50}) was 215.55 ± 6.29 μM,

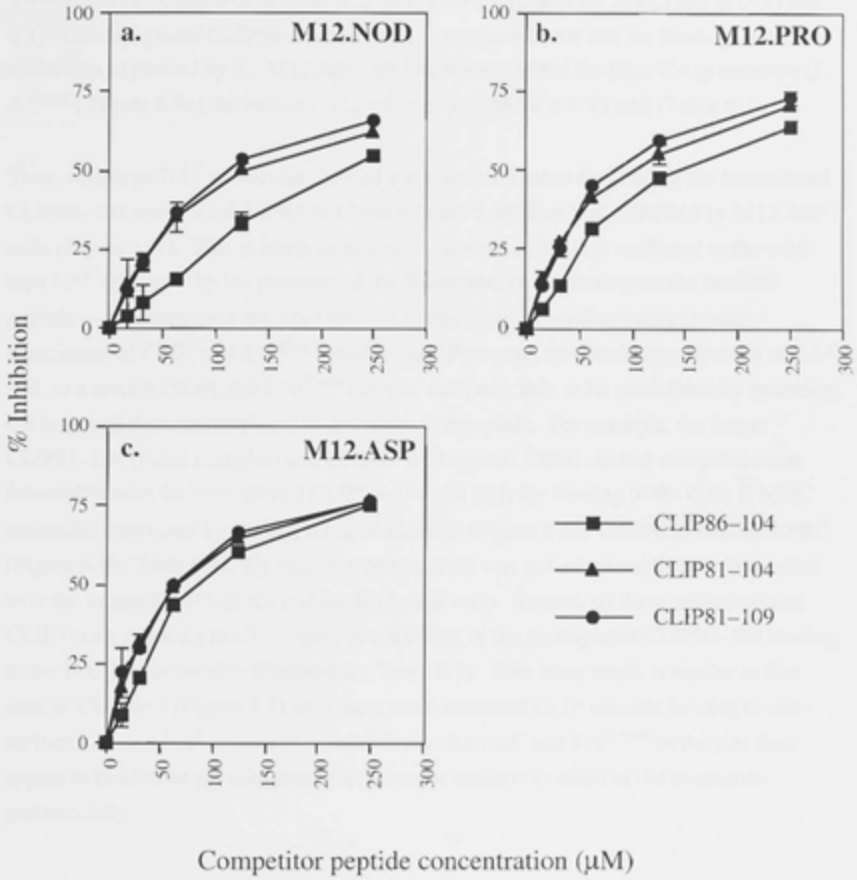


Figure 6.4 *Competition by CLIP ligands for cell-surface binding to mutated I-A^{g7} class II MHC molecules.*

The 19-mer, CLIP86–104, and its two extended variants, CLIP81–104 (24-mer) and CLIP81–104 (29-mer) were examined for their ability to inhibit the binding of biotinylated CLIP86–104 (50 μM) to the M12.A3-derived cell lines, **(a)** M12.NOD (wild-type I-A^{g7}), **(b)** M12.PRO (I-A^{g7Pro56}) and **(c)** M12.ASP (I-A^{g7Asp57}). Data shown represent the mean percentage inhibition ± SD from six independent experiments. Before calculating % inhibition, median fluorescence intensity (MFI) measurements were normalised for any minor differences in the levels of class II MHC expression between these cell lines, as determined by staining with OX6-FITC. Cells were incubated with biotinylated peptide and competitor for 18 hours at 37°C before flow cytometric analysis, as described in *Chapter 2*, §2.7.2.

a 4-fold excess, compared with an IC_{50} of 141.15 ± 4.03 μM for M12.PRO cells (Table 6.1). Unbiotinylated CLIP86–104 was able to compete better still for binding to the I-A^{g7} molecules expressed by the M12.ASP cell line that exhibited the βSer57Asp mutation (I-A^{g7Asp57}; Figure 6.4c), to yield an IC_{50} value of just 88.56 ± 1.95 μM (Table 6.1).

Thus, wild-type I-A^{g7} molecules showed a greater preference for binding the biotinylated CLIP86–104 over its unbiotinylated form (Figure 6.4a) than that exhibited by M12.ASP cells (Figure 6.4c). This is likely to reflect an increase in affinity conferred to the wild-type I-A^{g7} αβ dimer by the presence of the N-terminal biotin moiety on the labelled peptide — an interaction that does not add further positive binding energy to the association of CLIP with I-A^{g7Asp57} molecules. However, this binding preference of I-A^{g7} and, to a certain extent, the I-A^{g7Pro56} form as well, was able to be modulated by extending the length of the unbiotinylated CLIP competitor peptide. For example, the longer CLIP81–104 (filled triangles) and CLIP81–109 ligands (filled circles) competed more favourably with the biotinylated CLIP86–104 (50 μM) for binding to the class II MHC molecules expressed by the cell lines, M12.NOD (Figure 6.4a; Table 6.1) and M12.PRO (Figure 6.4b; Table 6.1). By contrast, competition was not enhanced to the same extent with the longer CLIP ligands and the M12.ASP cells. Instead, all three unbiotinylated CLIP forms yielded a similar degree of inhibition of the biotinylated CLIP86–104 binding to the I-A^{g7Asp57} molecules (Figure 6.4c; Table 6.1). This latter result is similar to that seen in *Chapter 3* (Figure 3.1) with these same extended CLIP variants binding to cell-surface I-A^{k} and I-A^{d} molecules. Collectively, the I-A^{g7} and I-A^{g7Pro56} molecules thus appear to bind those peptides that offer the most contacts to stabilise the interaction preferentially.

6.3.3 C-terminal residues of the CLIP86–104 ligand are critical for binding to I-A^{g7}

To investigate further the capacity of the I-A^{g7} β-chain mutations at positions 56 and 57 to affect the binding of peptides to this class II MHC variant, the specific features of the CLIP sequence necessary for interacting with this molecule were evaluated and compared with those of the single amino acid revertant forms, I-A^{g7Pro56} and I-A^{g7Asp57}. Specifically, the same series of substituted and/or length-altered CLIP analogues as used successfully in *Chapters 3–5* to define CLIP binding motifs for mouse I-A and I-E class II MHC molecules, were assessed for their abilities to compete against biotinylated CLIP86–104 (50 μM) for binding to the surface I-A molecules of the M12.NOD, M12.PRO and M12.ASP cell lines.

Table 6.1 *Competition by extended CLIP ligands for binding to mutated I-A^{g7} class II MHC molecules.*

CLIP		IC_{50} (μM)		
		M12.NOD	**M12.PRO**	**M12.ASP**
86–104	KPVSQMRMATPLLMRPMSM	215.55 ± 6.29	141.15 ± 4.03	88.56 ± 1.95
81–104	LPKSAKPVSQMRMATPLLMRPMSM	131.75 ± 14.78	96.19 ± 6.38	68.84 ± 4.53
81–109	LPKSAKPVSQMRMATPLLMRPMSMDNMLL	115.50 ± 0.42	86.42 ± 5.23	66.03 ± 5.40

Longer CLIP ligands were tested at multiple doses as competitors against biotinylated CLIP86–104 (50 μM) for binding to the cell-surface I-A class II MHC molecules expressed by the M12.A3-derived cell lines, M12.NOD (wild-type I-A^{g7}), M12.PRO (I-A^{g7Pro56}) and M12.ASP (I-A^{g7Asp57}). Data are presented as mean IC_{50} values (μM) from two independent experiments. Between experiments, mean background MFI (median fluorescence intensity) of 3.72, 3.00 and 3.01 relative units were recorded for each cell line, respectively, with mean maximum MFI measurements in the absence of competitor of 21.78, 22.06 and 26.84 relative units, respectively.

Firstly, the extent to which atoms of the CLIP backbone participate in bonds with the residues of I-A^{g7} and its different forms, I-A^{g7Pro56} and I-A^{g7Asp57}, was examined by determining the binding ability of D-alanine-substituted CLIP86–104 analogues listed previously in *Chapter 4*, Table 4.1. The results of one such assay, representative of the experimental findings, are shown in Figure 6.5. Mean IC_{50} values, calculated from two independent experiments are given atop each bar to provide a quantitative representation of the overall trends observed, *i.e.* the concentration of competitor peptide required to inhibit 50% of the biotinylated CLIP86–104 signal.

In common with the findings for mouse class II MHC molecules of both I-A and I-E isotypes in *Chapter 4* (Figures 4.5–4.6) and *Chapter 5* (Figure 5.7), respectively, the ability of these configurationally-altered ligands to bind to the three different I-A^{g7} variants was compromised severely when any of these substitutions fell in the central region of the peptide between residues Met 91 and Arg 100. However, unique to these I-A^{g7}-expressing cells, binding was also impaired considerably by the presence of a single D-alanine residue within the CLIP sequence C-terminal to this region. For example, D-alanine substitutions between Pro 101–Met 102 abrogated completely the binding to the wild-type I-A^{g7} and I-A^{g7Pro56} molecules, while the binding of the D-alanine-substituted Ser 103 analogue was reduced also (Figure 6.5a, b). By contrast, the ability of these C-terminal configurational inversions to disrupt binding appeared less severe with the I-A^{g7Asp57} class II MHC variant (Figure 6.5c). In particular, some degree of competition with the biotinylated CLIP was still observed with the configurationally-substituted Pro 101 and Met 102 analogues for binding to I-A^{g7Asp57}. Together, these results indicate that the typical conformation-dependent contacts between the backbone atoms of the central CLIP residues, 91–100, and the I-A^{g7} $\alpha\beta$ dimer are critically important for maintaining this interaction. However, additional contacts between I-A^{g7} and the C-terminus of this peptide (residues 101–103) are also needed to stabilise the binding, the requirement for which appears to be influenced to some extent by the replacement of an aspartic acid at position 57 in the β-chain of the I-A^{g7} heterodimer with serine (Figure 6.5c). A similar effect was not observed when the histidine at position β56 in wild-type I-A^{g7} was substituted by proline (Figure 6.5b).

Next, peptide side-chain contributions to the interaction between CLIP and the I-A^{g7} heterodimer and its mutated forms, I-A^{g7Pro56} and I-A^{g7Asp57}, were assessed using the series of L-alanine-substituted CLIP86–104 analogues listed previously in *Chapter 3*, Table 3.1. Figure 6.6 shows the results of one such assay, representative of the experimental findings. Again, mean IC_{50} values, calculated from two independent experiments, are given atop each bar to provide a quantitative representation of the overall

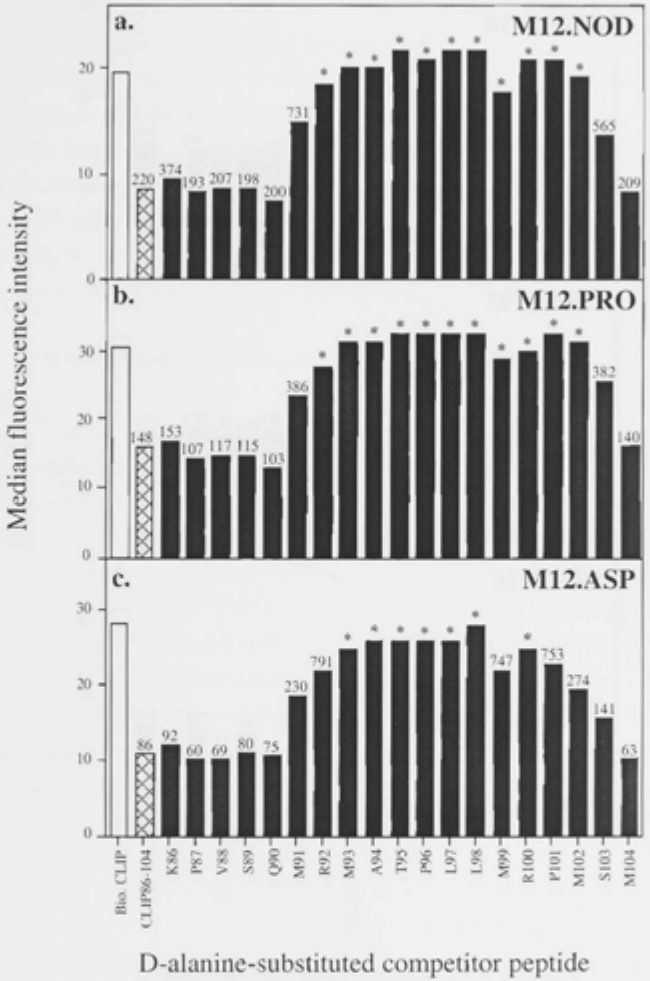


Figure 6.5 *Competition by D-alanine-substituted CLIP for cell-surface binding to mutated I-A^{g7} class II MHC molecules.*

Multiple doses of CLIP86–104 analogues with single D-alanine substitutions were tested as competitors against biotinylated CLIP86–104 (50 μM) for binding to cell-surface I-A molecules of the cell lines **(a)** M12.NOD, **(b)** M12.PRO and **(c)** M12.ASP. A single competitor dose which yielded ~50% inhibition of the biotinylated CLIP signal by wild-type CLIP86–104 (cross-hatched fill pattern) is shown (M12.NOD, 250 μM, M12.PRO & M12.ASP, 125 μM). All assays were performed twice with consistent results. Solid bars represent competition by the substituted CLIP and empty bars indicate the maximal biotinylated CLIP signal when no competitor is present. Binding is expressed as median fluorescence intensity (MFI), from which background counts measured in the absence of biotinylated peptide have been subtracted (M12.NOD, 2.87; M12.PRO, 3.22; M12.ASP, 2.69).

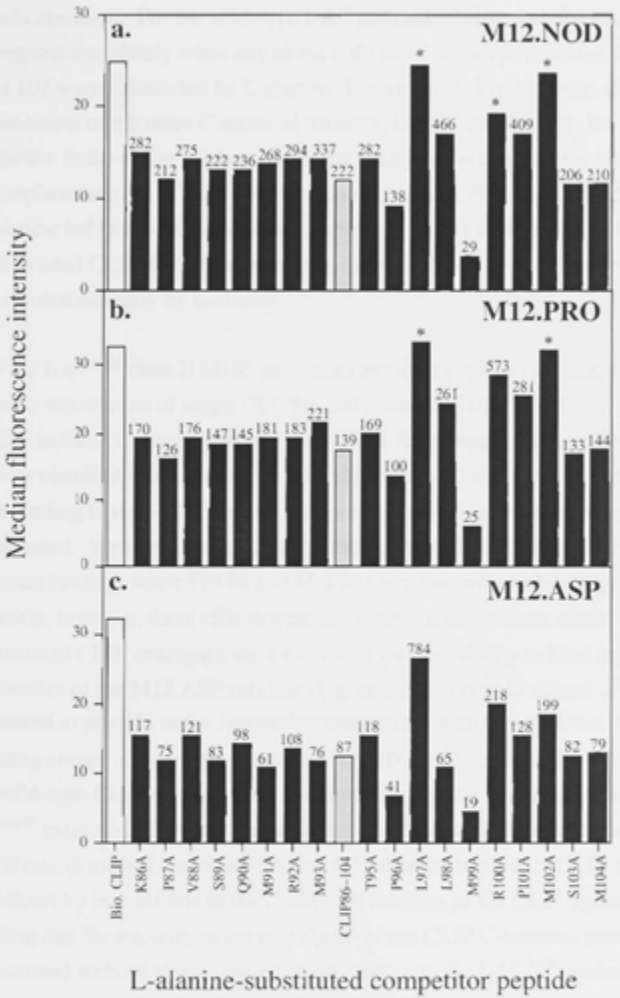


Figure 6.6 *Competition by L-alanine-substituted CLIP86–104 for cell-surface binding to mutated I-A^{g7} class II MHC molecules.*

Multiple doses of CLIP86–104 analogues with single L-alanine substitutions were tested as competitors against biotinylated CLIP86–104 (50 μM) for binding to the cell-surface I-A molecules of the cell lines **(a)** M12.NOD, **(b)** M12.PRO and **(c)** M12.ASP. A single competitor dose which yielded ~50% inhibition of the biotinylated CLIP signal by wild-type CLIP86–104 (shaded fill pattern) is shown (M12.NOD, 250 μM; M12.PRO & M12.ASP, 125 μM). All assays were performed twice with equivalent results. Solid bars represent competition by the substituted CLIP and empty bars indicate the maximal biotinylated CLIP signal with no competitor peptide. Binding is expressed as median fluorescence intensity (MFI), from which background counts, measured in the absence of biotinylated peptide, have been subtracted (M12.NOD, 2.92 ; M12.PRO, 3.16; M12.ASP, 2.94).

trends observed. For the wild-type I-A^{g7} molecule, binding of the CLIP sequence was abrogated completely when any of the individual wild-type residues, Leu 97, Arg 100 and Met 102 were substituted by L-alanine (Figure 6.6a). Furthermore, the single substitution of the other C-terminal residues, Leu 98 and Pro 101, led to a significant reduction in the ability of these particular analogues to compete for binding. Meanwhile, the replacement of the methionine residue at position 99 in the CLIP86–104 sequence by L-alanine led to a marked increase in the ability of this analogue to compete with the biotinylated CLIP86–104. A smaller increase in binding was observed when Pro 96 was substituted similarly by L-alanine.

For the I-A^{g7Pro56} class II MHC molecules expressed by the cell line, M12.PRO, the L-alanine substitution of single CLIP86–104 residues yielded an almost identical pattern of results to those obtained with the wild-type I-A^{g7} molecule (Figure 6.6b). For example, anchor contributions provided by the residues Leu 97 and Met 102 were identified, such that binding to the I-A^{g7Pro56} molecules was not sustained when these residues were substituted. Similarly, the L-alanine substitution of Leu 98, Arg 100 and Pro 101 also reduced binding, while Pro 96 and Met 99 were somewhat inhibitory to the interaction. Notably, however, these effects were not observed to the same extent when the L-alanine-substituted CLIP analogues were examined for their ability to bind to the I-A^{g7Asp57} molecules of the M12.ASP cell line (Figure 6.6c). For this variant of I-A^{g7}, only Leu 97 appeared to provide major favourable interactions with the $\alpha\beta$ dimer, with some positive binding energy also being provided by Arg 100 and Met 102. Again, the Met 99 and Pro 96 wild-type CLIP residues were both inhibitory to the binding of this sequence to the I-A^{g7Asp57} molecule. Together, these results confirm the findings obtained with the backbone-disrupted analogues (Figure 6.5), that the binding of CLIP to I-A^{g7} is critically stabilised by interactions of the C-terminal residues of the CLIP ligand. Furthermore, the finding that the majority of the side chains of the CLIP C-terminal residues may be substituted without significant effect on binding to the I-A^{g7Asp57} molecules adds conviction to the idea that the need for such additional interactions arises from the absence of an aspartic acid residue at position 57 in the I-A^{g7} β-chain (Figure 6.5c). By contrast, the lack of proline at position β56 did not show such an effect (Figure 6.5b).

To investigate further the importance of the C-terminal CLIP residues in stabilising the interaction of this ligand with I-A^{g7}, the binding of a series of frameshifted 15-mer CLIP analogues to this class II MHC molecule was assessed in a competitive cell-surface assay (Table 6.2). These ligands have been used previously to determine the importance of specific N-terminal CLIP residues for binding to I-A and I-E molecules (*Chapter 4*, Table 4.3 & *Chapter 5*, Table 5.2). Data presented in Table 6.2 depict the mean concentration

Table 6.2 *Competition by frameshifted CLIP 15-mers for binding to mutated I-A^{g7} class II MHC molecules.*

CLIP		IC_{50} (μM)		
		M12.NOD	**M12.PRO**	**M12.ASP**
86–100	KPVSQMRMATPLLMR	> 1000	> 1000	246.40 ± 5.94
87–101	PVSQMRMATPLLMRP	> 1000	> 1000	265.65 ± 22.42
88–102	VSQMRMATPLLMRPM	468.45 ± 2.19	478.30 ± 135.91	166.40 ± 7.50
89–103	SQMRMATPLLMRPMS	260.75 ± 40.09	200.30 ± 1.41	126.40 ± 1.27
90–104	QMRMATPLLMRPMSM	300.00 ± 7.64	215.25 ± 39.67	148.60 ± 5.94

Fifteen-residue CLIP variants were synthesised, each with a single amino-acid frameshift. Analogues were tested at multiple doses as competitors against biotinylated CLIP86–104 (50 μM) for binding to the cell-surface I-A class II MHC molecules expressed by the M12.A3-derived cell lines, M12.NOD (wild-type I-A^{g7}), M12.PRO (I-A^{g7Pro56}) and M12.ASP (I-A^{g7Asp57}). Data are presented as mean IC_{50} values (μM) from two independent experiments. Mean background MFI measurements and maximum signals recorded in the absence of competitors are as in Table 6.1.

of these frameshifted CLIP required to yield 50% inhibition (IC_{50}) of the biotinylated CLIP86–104 signal (50 μM) for each of the cell lines, M12.NOD, M12.PRO and M12.ASP, calculated from two independent experiments. From these results, it is apparent that CLIP binding to I-A^{g7} is extremely sensitive to any frameshifts which result in the loss of the C-terminal CLIP residues, Pro 101 & Met 102. For example, the peptides, CLIP86–100 and 87–101, are unable to compete for binding to I-A^{g7} while CLIP88–102, binds poorly. This further confirms the importance of the C-terminal region of the CLIP ligand for binding to I-A^{g7} noted earlier in the backbone (Figure 6.5) and side-chain substitution assays (Figure 6.6). By contrast, truncations at the N-terminus that leave these C-terminal residues intact (CLIP89–103 and 90–104) have very little effect upon the binding affinity. In this respect, it is interesting to note that this pattern of results is in distinct contrast to the findings obtained in *Chapter 4* (Table 4.3), with these same peptides binding to other I-A allotypes, I-A^{a}, I-A^{k} and I-A^{d}. There, those CLIP ligands that contained all or part of the original N-terminal sequence were found to bind better than those that exhibited the corresponding C-terminal residues.

As with the previous side-chain and backbone substitution assays, an identical pattern of results is obtained between wild-type I-A^{g7} and I-A^{g7Pro56} molecules for the binding of the frameshifted CLIP analogues (Table 6.2). Also as seen before, the pattern of binding indicating the importance of the CLIP C-terminal residues is not as marked with the I-A^{g7Asp57} molecules expressed by the M12.ASP cells. For example, CLIP86–100 still competes well with biotinylated CLIP for binding to I-A^{g7Asp57}. This supports further the notion that the loss of the aspartic acid residue at position 57 in the β-chain of the wild-type I-A^{g7} is a contributory factor in the need for additional interactions to form with the C-terminal CLIP residues in order to stabilise binding. Nevertheless, the pattern of these frameshifted peptides binding to I-A^{g7Asp57} is still overall the same as that observed for I-A^{g7} and I-A^{g7Pro56}, in that CLIP90–104 binds better to I-A^{g7Asp57} than CLIP86–100 (compare with *Chapter 4*, Table 4.3). This indicates that the lack of aspartic acid at position 57 in the β-chain of the I-A^{g7} molecule is not the sole feature which is responsible for the cluster of residues at the C-terminus of the CLIP ligand providing the unusual anchor contributions.

Next, the minimum length of the CLIP ligand required for binding to the I-A^{g7} αβ dimer and each of its variant forms, I-A^{g7Pro56} and I-A^{g7Asp57}, was determined using a set of peptides truncated by a single residue at each end from the wild-type CLIP86–104 sequence, as shown in Table 6.3. As with the frameshifted peptides examined above (Table 6.2), data are presented as the mean concentration of truncated peptide required to yield 50% inhibition (IC_{50}) of the biotinylated CLIP86–104 signal (50 μM) for each of the

Table 6.3 *Competition by truncated CLIP86–104 for binding to mutated I-A^{g7} class II MHC molecules.*

CLIP		IC_{50} (μM)		
		M12.NOD	**M12.PRO**	**M12.ASP**
86–104	KPVSQMRMATPLLMRPMSM	215.55 ± 6.29	141.15 ± 4.03	88.56 ± 1.95
87–103	PVSQMRMATPLLMRPMS	184.05 ± 31.89	141.15 ± 32.88	86.62 ± 0.31
88–102	VSQMRMATPLLMRPM	468.45 ± 2.19	478.30 ± 135.91	166.40 ± 7.50
89–101	SQMRMATPLLMRP	> 1000	> 1000	474.45 ± 21.57
90–100	QMRMATPLLMR	> 1000	> 1000	> 1000
91–99	MRMATPLLM	> 1000	> 1000	> 1000

Residues were removed pairwise, sequentially from each end of the CLIP86–104 sequence. The truncated CLIP were tested at multiple doses as competitors against biotinylated CLIP86–104 (50 μM) for binding to the cell-surface I-A class II MHC molecules of the M12-A3-derived cell lines, M12.NOD (wild-type I-A^{g7}), M12.PRO (I-A^{g7Pro56}) and M12.ASP (I-A^{g7Asp57}). Data are presented as mean IC_{50} values (μM) from two independent experiments. Mean background MFI measurements and maximum signals recorded in the absence of competitors are as in Table 6.1.

cell lines, M12.NOD, M12.PRO and M12.ASP, calculated from two independent experiments. For the wild-type I-A^{g7} molecule, the effect of truncating the CLIP ligand below 15 residues was severe, with no binding recorded whatsoever for the peptides CLIP89–101, 90–100 or 91–99. Similarly for I-A^{g7Pro56}, any competition against the biotinylated CLIP86–104 peptide by these shorter CLIP was unable to be measured. For I-A^{g7Asp57}, however, these progressive pairwise truncations caused a more gradual deterioration in the CLIP binding capacity, such that some inhibition of the biotinylated CLIP86–104 signal was still able to be detected with the 13-mer CLIP89–101 peptide. This latter result is reminiscent of those obtained with other allotypes of the I-A class II MHC molecule, I-A^{u}, I-A^{k} and I-A^{d}, using this same experimental approach (*Chapter 4*, Table 4.2).

Lastly in this series of experiments, the contribution of the particular amino acid residues that flank the central sequence element, CLIP91–99, to the interaction with the molecules, I-A^{g7}, I-A^{g7Pro56} and I-A^{g7Asp57}, was explored further by evaluating the binding abilities of another set of CLIP86–104 analogues, this time exhibiting a number of different truncations and/or L-alanine substitutions. Shown in Table 6.4 are the results of these peptides competing against biotinylated CLIP86–104 for binding to the cell-surface I-A molecules of the cell lines, M12.NOD, M12.PRO and M12.ASP. Again, data are presented as mean IC_{50} values ± standard deviation from two independent experiments. Significant effects upon binding where not observed by sequence modifications at the N-terminus of the CLIP ligand, *e.g.* CLIP87–104, 87A–104, consistent with these side chains not participating in favourable interactions with the three I-A^{g7} variants (Figure 6.6). However, of particular interest, the binding of CLIP to I-A^{g7} and I-A^{g7Pro56} molecules was impaired significantly when the residues Ser 103 & Met 104 were deleted (*i.e.* CLIP87–102). This is consistent with the side chain of Met 102 forming critical anchor contacts with these class II MHC molecules (Figure 6.6). In this context, it is envisaged that this anchor interaction might be weakened when this residue is not flanked by at least one other amino acid. By comparison, the binding of the CLIP ligand to I-A^{g7Asp57} molecules was not affected as much by such modifications, consistent with the lesser contribution of the Met 102 residue to stabilising this interaction. It would also appear that the Ser 103 residue may provide some negative binding energy to the association of CLIP with the I-A^{g7} αβ dimer, as indicated by the increase in binding observed when this residue was substituted by L-alanine (*e.g.* Ala-CLIP, 86–103A). Alternatively, this may be the result of effects transmitted upon the neighbouring anchor residue, Met 102. Consistent with this, L-alanine substitution of the Ser 103 residue in the wild-type CLIP86–104 sequence did not exhibit any effect upon binding affinity (Figure 6.6).

Table 6.4 *Competition by substituted and/or truncated CLIP86–104 for binding to mutated I-A^{g7} class II MHC molecules.*

CLIP		IC_{50} (μM)		
		M12.NOD	**M12.PRO**	**M12.ASP**
86–104	KPVSQMRMATPLLMRPMSM	215.55 ± 6.29	141.15 ± 4.03	88.56 ± 1.95
Ala-CLIP	AAVSQMRMATPLLMRPMAA	160.25 ± 9.69	115.60 ± 15.41	68.47 ± 5.13
86–103	KPVSQMRMATPLLMRPMS	224.45 ± 10.96	150.25 ± 24.25	95.83 ± 1.20
86–103A	KPVSQMRMATPLLMRPMA	132.70 ± 12.87	86.03 ± 6.16	56.64 ± 0.04
87–104	PVSQMRMATPLLMRPMSM	213.15 ± 7.57	180.10 ± 26.59	100.25 ± 4.46
87A–104	AVSQMRMATPLLMRPMSM	227.00 ± 6.65	159.10 ± 12.45	108.10 ± 2.97
87–102	PVSQMRMATPLLMRPM	476.75 ± 63.57	542.95 ± 123.67	134.05 ± 10.39
88–103	VSQMRMATPLLMRPMS	189.65 ± 2.19	148.40 ± 9.62	92.81 ± 4.52

Substituted and/or truncated CLIP86–104 analogues were tested at multiple doses as competitors against biotinylated CLIP86–104 (50 μM) for binding to the cell-surface I-A class II MHC molecules expressed by the M12.A3-derived cell lines, M12.NOD (wild-type I-A^{g7}), M12.PRO (I-A^{g7Pro56}) and M12.ASP (I-A^{g7Asp57}). Data are presented as mean IC_{50} values (μM) from two independent experiments. Mean background MFI measurements and maximum signals recorded in the absence of competitors are as in Table 6.1.

6.3.4 Serine at position β57 in the I-A^{g7} MHC molecule bestows a preference for peptide ligands that exhibit an acidic residue at P9

The β-chain mutations, βPro56His and βAsp57Ser, in I-A^{g7} both have the potential to introduce preferences for negatively-charged side chains within the C-terminus of I-A^{g7}-binding peptides (discussed previously in §6.1.5). For example, the βAsp57Ser mutation in I-A^{g7} is predicted to confer a preference for an acidic residue at relative position P9 by consequence of the nearby unpaired positive charge of αArg 76 (Brown *et al.*, 1993; Reich *et al.*, 1994; Stern *et al.*, 1994). An acidic residue which might interact with the βHis 56 residue of I-A^{g7}, however, would be expected to be C-terminal to this position, perhaps at P10 or P11 given the location of the β56 residue at the very edge of the binding cleft (Brown *et al.*, 1993; Stern *et al.*, 1994; Ghosh *et al.*, 1995; Fremont *et al.*, 1996, 1998b; Scott *et al.*, 1998). To examine further the possible interactions of acidic residues with the β56 and β57 residues of the I-A^{g7} peptide-binding groove, a number of known ligands of this αβ dimer were examined for their ability to bind to the cell-surface molecules expressed by the four cell lines, M12.A3, M12.NOD, M12.PRO and M12.ASP.

The sequences of the mouse serum albumin peptide, MSA560–574, and the determinant, p12(166–185), from the mouse heat shock protein 60 (HSP60), are shown in Figure 6.7a and 6.7b, respectively. Boxed amino acids indicate the 9-residue binding frame corresponding to the stretch of peptide likely to be accommodated within the I-A^{g7} binding groove, as defined by the studies of Reich *et al.* (1994) and Reizis *et al.* (1997b), respectively. Notably, both of these I-A^{g7}-binding peptides exhibit an acidic residue in the form of glutamic acid at the P9 position (shown in bold), with a second C-terminal glutamic acid at position P10 (MSA) or P11 (p12).

Representative experiments of the binding of biotinylated MSA560–574 and p12(166–185) to the cell-surface class II MHC molecules of the cell lines, M12.A3, M12.NOD, M12.PRO and M12.ASP, are shown in Figures 6.7c and 6.7d, respectively. As performed previously with the binding of biotinylated CLIP ligands to these cell lines in Figure 6.3, adjustments have been made in the levels of median fluorescence intensity (MFI) for any variations in the expression of the I-A^{g7} class II MHC molecules. Both MSA and p12 peptides bound well to the wild-type I-A^{g7} expressed at the surface of the M12.NOD cells (filled squares). This binding is specific for these class II MHC molecules on this cell line, as shown by the negligible associations recorded with the parental line, M12.A3, which expresses only I-E^{d} (open squares).

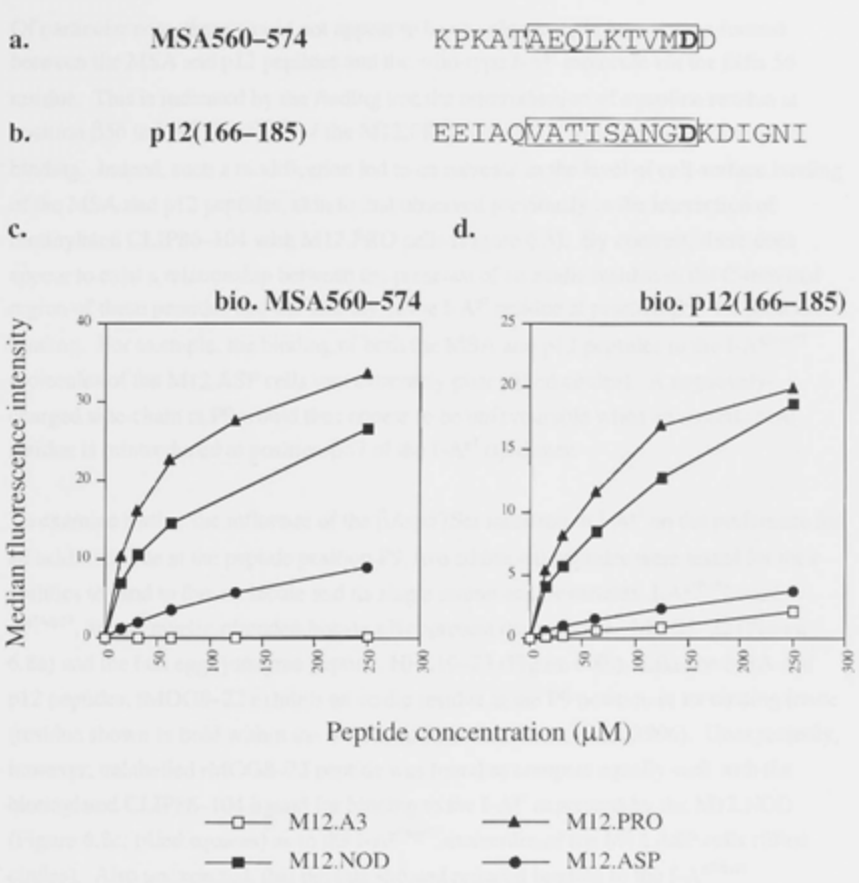


Figure 6.7 *The binding of antigen-derived peptides to mutated I-A^{g7} class II MHC molecules.*

Biotinylated peptides **(a)** MSA560–574, from mouse serum albumin, and **(b)** p12(166–185), from mouse heat shock protein 60, were examined for their ability to bind to the mutated I-A^{g7} class II MHC molecules expressed on the surface of the M12.A3-derived cell lines, M12.PRO (I-Ag^{7Pro56}) and M12.ASP (I-Ag^{7Asp57}). Binding was compared to that shown with the M12.NOD cell line, also derived from M12.A3 but which expresses wild-type I-A^{g7}. In **(c)** and **(d)** are shown one of three independent experiments for each peptide. The level of non-specific binding of these peptides is indicated by the M12.A3 parental cell line. Binding is expressed as median fluorescence intensity (MFI) from which background signals, measured in the absence of biotinylated peptide, have been subtracted (M12.A3, 3.40; M12.NOD, 2.69; M12.PRO, 3.05; M12.ASP, 2.64). MFI measurements have also been normalised for any minor differences in the levels of class II MHC expression between these cell lines, as determined by staining with OX6-FITC. Cells were incubated with biotinylated peptide for 18 hours at 37°C before flow cytometric analysis, as described in *Chapter 2*, §2.7.2.

Of particular note, there would not appear to be any electrostatic interactions formed between the MSA and p12 peptides and the wild-type I-A^{g7} molecule *via* the βHis 56 residue. This is indicated by the finding that the reintroduction of a proline residue at position β56 in I-A^{g7} (I-A^{g7Pro56} of the M12.PRO cells; filled triangles) did not impair binding. Indeed, such a modification led to an increase in the level of cell-surface binding of the MSA and p12 peptides, akin to that observed previously in the interaction of biotinylated CLIP86–104 with M12.PRO cells (Figure 6.3). By contrast, there does appear to exist a relationship between the presence of an acidic residue in the C-terminal region of these peptides and the identity of the I-A^{g7} residue at position β57 for productive binding. For example, the binding of both the MSA and p12 peptides to the I-A^{g7Asp57} molecules of the M12.ASP cells was extremely poor (filled circles). A negatively-charged side-chain at P9 would thus appear to be unfavourable when an aspartic acid residue is reintroduced at position β57 of the I-A^{g7} αβ dimer.

To examine further the influence of the βAsp57Ser mutation in I-A^{g7} on the preference for an acidic residue at the peptide position P9, two additional peptides were tested for their abilities to bind to this molecule and its single amino acid revertants, I-A^{g7Pro56} and I-A^{g7Asp57}, the rat myelin oligodendrocyte glycoprotein determinant, rMOG8–22 (Figure 6.8a) and the hen egg lysozyme peptide, HEL10–23 (Figure 6.8b). Like the MSA and p12 peptides, rMOG8–22 exhibits an acidic residue at the P9 position in its binding frame (residue shown in bold within the boxed amino acids; Amor *et al.*, 1996). Unexpectedly, however, unlabelled rMOG8–22 peptide was found to compete equally well with the biotinylated CLIP86–104 ligand for binding to the I-A^{g7} expressed by the M12.NOD (Figure 6.8c; filled squares) as to the I-A^{g7Asp57} molecules of the M12.ASP cells (filled circles). Also unexpected, this peptide showed reduced binding to the I-A^{g7Pro56} molecules of the M12.PRO cell line (filled triangles).

In comparison, the HEL10–23 peptide does not exhibit an acidic residue at relative position P9 (residue shown in bold within the boxed amino acid binding frame; Harrison *et al.*, 1997). A representative experiment of the binding of the biotinylated HEL peptide with the cell lines, M12.A3, M12.NOD, M12.PRO and M12.ASP, is shown in Figure 6.8d, with MFI levels again adjusted for any variations in class II MHC expression between cell lines. As found for the MSA and p12 peptides previously (Figure 6.7), binding occurs specifically to the I-A^{g7} molecules expressed by the M12.NOD cell line (filled squares) and not to I-E^{d} also present on these cells, as indicated by the lack of fluorescence signal detected with the parental cell line, M12.A3 (open squares). Moreover, the binding of the HEL peptide to I-A^{g7} indicates that the presence of an acidic residue at the peptide P9 position is not an absolute prerequisite for this interaction. The

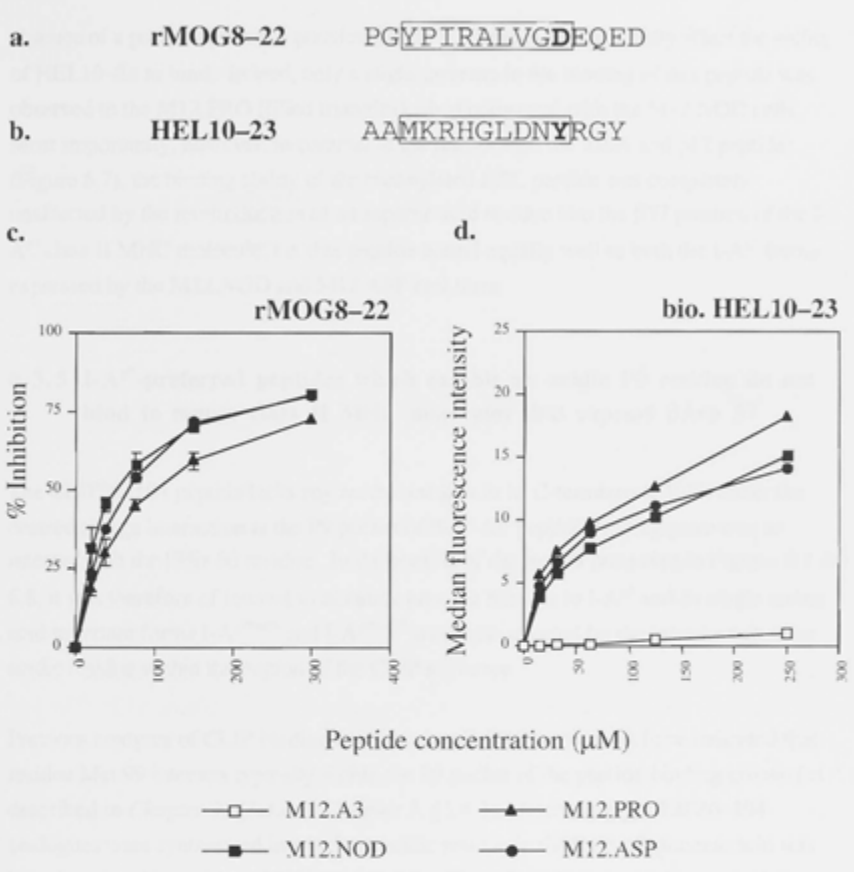


Figure 6.8 *The binding of antigen-derived peptides to mutated I-A^{g7} class II MHC molecules.*

The peptides **(a)** rMOG8–22, from rat myelin oligodendrocyte glycoprotein, and **(b)** HEL10–23, from hen egg lysozyme, were assessed for their ability to bind to the mutated I-A^{g7} class II MHC molecules expressed by the cell lines, M12.PRO (I-A^{g7Pro56}) and M12.PRO (I-A^{g7Asp57}). Briefly, **(c)** rMOG8–22 was tested as a competitor against biotinylated CLIP86–104 (50 μM) for cell-surface binding to M12.NOD, M12.PRO and M12.ASP cells. Data presented represent the mean percentage inhibition ± SD from two independent experiments. **(d)** Biotinylated HEL10–23 was assessed for its ability to bind directly to the M12.PRO and M12.ASP cells. Binding was compared to that obtained with M12.NOD, also derived from M12.A3 but which expresses wild-type I-A^{g7}. Shown is one of three independent experiments, all yielding equivalent results. For the HEL peptide, binding is expressed as median fluorescence intensity (MFI) and non-specific associations are indicated by the M12.A3 parental cell line. For both **(c)** and **(d)**, background signal, as determined in the absence of biotinylated peptide, has been subtracted from data (M12.A3, 3.40; M12.NOD, 2.69; M12.PRO, 3.05; M12.ASP, 2.64). MFI measurements have also been normalised for any minor differences in the levels of class II MHC expression between these cell lines, as determined by OX-6-FITC staining. Cells were incubated with biotinylated peptide (± competitor) for 18 hours at 37°C before flow cytometric analysis, as described in *Chapter 2*, §2.7.2.

absence of a proline residue at position β56 in I-A^{g7} did not significantly affect the ability of HEL10–23 to bind. Indeed, only a slight increase in the binding of this peptide was observed to the M12.PRO (filled triangles) when compared with the M12.NOD cells. Most importantly, however, in contrast to the results with the MSA and p12 peptides (Figure 6.7), the binding ability of the biotinylated HEL peptide was completely unaffected by the reintroduction of an aspartic acid residue into the β57 position of the I-A^{g7} class II MHC molecule, *i.e.* this peptide bound equally well to both the I-A^{g7} forms expressed by the M12.NOD and M12.ASP cell lines.

6.3.5 I-A^{g7}-preferred peptides which exhibit an acidic P9 residue do not bind to mouse class II MHC molecules that express βAsp 57

The CLIP86–104 peptide lacks any acidic residues in its C-terminus to fulfil either the desired charge interaction at the P9 pocket of the I-A^{g7} peptide-binding groove or to interact with the βHis 56 residue. In the context of the results presented in Figures 6.7 & 6.8, it was therefore of interest to examine how the binding to I-A^{g7} and its single amino acid revertant forms I-A^{g7Pro56} and I-A^{g7Asp57} would be affected by the introduction of an acidic residue within this region of the CLIP sequence.

Previous analyses of CLIP binding to other class II MHC molecules have indicated that residue Met 99 interacts typically within the P9 pocket of the peptide-binding groove (as described in *Chapter 3*, §3.4.4 & *Chapter 5*, §5.4.2). Accordingly, CLIP86–104 analogues were synthesised in which an acidic residue in the form of glutamic acid was introduced at this position (M99E; Table 6.5). The effect of such a residue was also tested at position 100 (R100E) within the CLIP86–104 sequence, both as a single amino-acid substitution (R100E) and together with the replacement of the Met 99 side chain for an alanine residue (M99A/R100E). This latter peptide was synthesised in the event that the presence of methionine at position 99 in the single-substituted peptide was inhibitory to such an extent (as shown in Figure 6.6), that it masked any positive interactions made with an acidic residue at position 100. These three peptides were then tested alongside wild-type CLIP86–104 and the singly-substituted M99A peptide for their abilities to compete against biotinylated wild-type CLIP86–104 for binding to the forms of the I-A^{g7} molecule expressed by the cell lines, M12.NOD, M12.PRO and M12.ASP (Figure 6.9). Data are presented as mean percentage inhibition of the biotinylated CLIP signal from two independent experiments.

Table 6.5 *Met 99 and/or Arg 100-substituted CLIP86–104 analogues*

wild-type	K	P	V	S	Q	M	R	M	A	T	P	L	L	M	R	P	M	S	M		
M99A	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-		
M99E	-	-	-	-	-	-	-	-	-	-	-	-	-	E	-	-	-	-	-		
R100E	-	-	-	-	-	-	-	-	-	-	-	-	-	-	E	-	-	-	-		
M99A/R100E	-	-	-	-	-	-	-	-	-	-	-	-	-	A	E	-	-	-	-		
M99Q	-	-	-	-	-	-	-	-	-	-	-	-	-	Q	-	-	-	-	-		

Amino acid sequences of CLIP86–104 analogues bearing amino acid substitutions of residue 99 and/or 100. Native amino acid residues are represented with a dash.

As found previously in the L-alanine scan of the CLIP86–104 sequence in Figure 6.6, the M99A peptide (filled diamonds) again competed considerably better than the wild-type CLIP sequence (open squares) for binding to I-A^{g7} (Figure 6.9a), I-A^{g7Pro56} (Figure 6.9b) and I-A^{g7Asp57} (Figure 6.9c). The M99E peptide (filled circles) also competed extremely well for binding to I-A^{g7} and I-A^{g7Pro56} however showed a remarkably poor ability to bind to I-A^{g7Asp57}, reminiscent of the binding of the MSA560–574 and p12(166–185) peptides to this same class II MHC variant (Figure 6.7). This result thus confirms that the residue equivalent to Ii-position 99 within the CLIP sequence is indeed the residue that interacts at the P9 pocket of the peptide-binding groove of the I-A^{g7} $\alpha\beta$ dimer.

Consistent with the alignment of Met 99 with the P9 pocket of the I-A^{g7} groove, substitution of residue Arg 100 of the wild-type CLIP sequence for glutamic acid (R100E; filled triangles) did not create an analogue which bound well to I-A^{g7}. Indeed, the R100E peptide bound very poorly to both I-A^{g7} and I-A^{g7Pro56} (Figure 6.9). The L-alanine scans of the CLIP sequence shown in Figure 6.6 had not been completed at the time of designing this peptide or it would have been known that, in fact, the wild-type Arg 100 residue is an important C-terminal anchor for the binding of CLIP to I-A^{g7} molecules, and to a lesser extent, to I-A^{g7Pro56}. However, binding of the R100E peptide was slightly better to I-A^{g7Asp57} (Figure 6.9c). This is consistent with the wild-type Arg 100 residue providing less critical positive binding energy to stabilise the interaction between CLIP and this class II MHC variant (Figure 6.6c). Of particular interest, the removal of the favourable interaction between Arg 100 and the I-A^{g7} and I-A^{g7Pro56} heterodimers by the R100E substitution was able to be compensated for by the replacement of the inhibitory methionine residue at position 99 with the smaller alanine in the peptide, M99A/R100E

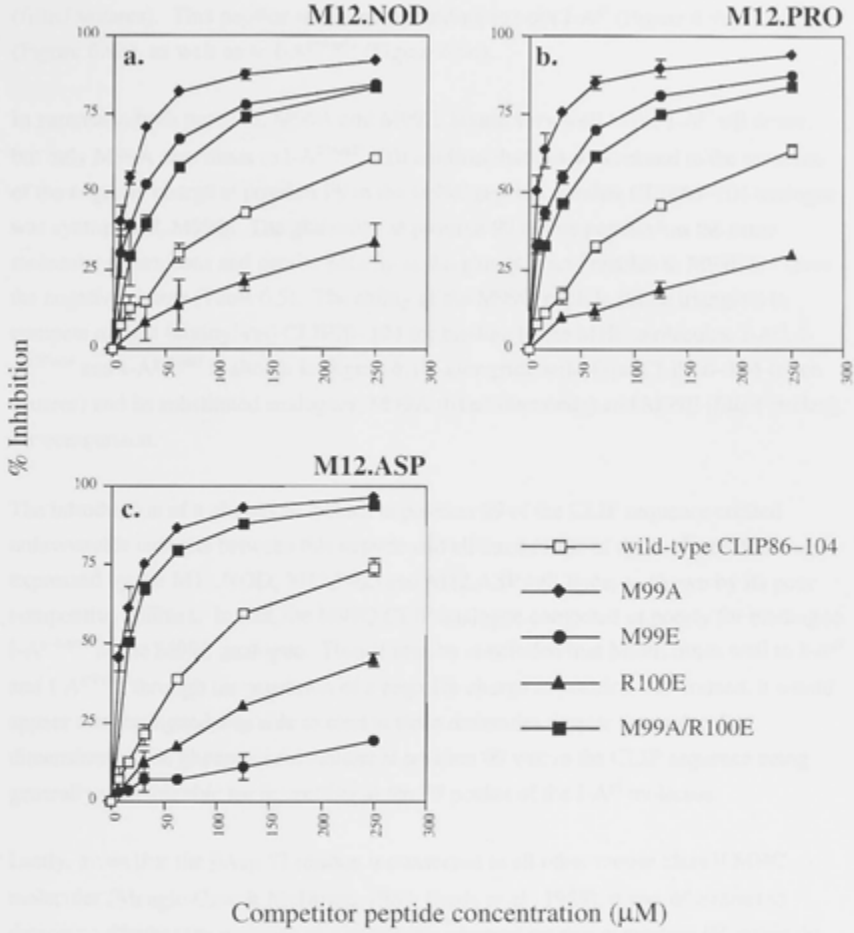


Figure 6.9 *Competition by substituted CLIP analogues for cell-surface binding to mutated I-A^{g7} class II MHC molecules.*

Substituted CLIP86–104 analogues were examined for their ability to inhibit the cell-surface binding of biotinylated CLIP86–104 (50 μM) to the M12.A3-derived cell lines, M12.PRO (I-A^{g7Pro56}) and M12.ASP (I-A^{g7Asp57}) which express mutated forms of the I-A^{g7} class II MHC molecule. Binding was compared to that obtained with M12.NOD cells, also derived from M12.A3 but which express wild-type I-A^{g7}. Data shown represent the mean percentage inhibition ± SD from six independent experiments. Before calulating % inhibition, median fluorescence intensity (MFI) measurements were normalised for any minor differences in the levels of class II MHC expression between these cell lines, as determined by OX6-FITC staining. Cells were incubated with biotinylated peptide and competitor for 18 hours at 37°C before flow cytometric analysis, as described in *Chapter 2*, §2.7.2.

(filled squares). This peptide shows good binding to both I-A^{g7} (Figure 6.9a), I-A^{g7Pro56} (Figure 6.9b), as well as to I-A^{g7Asp57} (Figure 6.9c).

In summary, both peptides, M99A and M99E, bound very well to the I-A^{g7} $\alpha\beta$ dimer, but only M99A also binds to I-A^{g7Asp57}. To confirm that this was related to the presence of the negative charge at position P9 in the M99E peptide, another CLIP86–104 analogue was synthesised, M99Q. The glutamine at position 99 in this peptide has the same molecular dimensions and similar polarity as the glutamic acid residue in M99E but lacks the negative charge (Table 6.5). The ability of the M99Q peptide (filled triangles) to compete against biotinylated CLIP86–104 for binding to the MHC molecules, I-A^{g7}, I-A^{g7Pro56} and I-A^{g7Asp57} is shown in Figure 6.10, alongside wild-type CLIP86–104 (open squares) and its substituted analogues, M99A (filled diamonds) and M99E (filled circles), for comparison.

The introduction of a glutamine residue at position 99 of the CLIP sequence created unfavourable contacts between this peptide and all three forms of the I-A^{g7} molecule expressed by the M12.NOD, M12.PRO and M12.ASP cell lines, as shown by its poor competitive abilities. In fact, the M99Q CLIP analogue competed as poorly for binding to I-A^{g7Asp57} as the M99E analogue. Thus it may be concluded that M99E binds well to I-A^{g7} and I-A^{g7Pro56} through the provision of a negative charge at position P9. Indeed, it would appear that this ligand was able to bind to these molecules despite the molecular dimensions of the glutamic acid residue at position 99 within the CLIP sequence being generally unfavourable for interacting at the P9 pocket of the I-A^{g7} molecule.

Lastly, given that the βAsp 57 residue is conserved in all other mouse class II MHC molecules (Mengle-Gaw & McDevitt, 1983; Davis *et al.*, 1989), it was of interest to determine whether the presence of a negatively-charged residue at position 99 within the CLIP sequence would destroy the binding of this ligand to these variants, as found for the βSer57Asp I-A^{g7} revertant, I-A^{g7Asp57} in Figures 6.7, 6.9 and 6.10. Shown in Figure 6.11, the peptides M99A, M99E and M99Q were examined alongside wild-type CLIP86–104 (open squares) for their ability to compete against biotinylated CLIP86–104 for binding to the cell-surface mouse class II MHC molecules, I-A^{d} (Figure 6.11a, b), I-A^{u} (Figure 6.11c) and I-A^{k} (Figure 6.11d). Data are presented as mean percentage inhibition ± standard deviation from two independent experiments. Compatible with the L-alanine scan reported in *Chapter 3* (Figure 3.6) and by Gautam *et al.*, 1995), the replacement of Met 99 by the smaller L-alanine residue (filled diamonds) led to an improvement of binding of this ligand to all three I-A variants examined. By contrast, the presence of a glutamine residue at this position was much less favourable to binding than both the

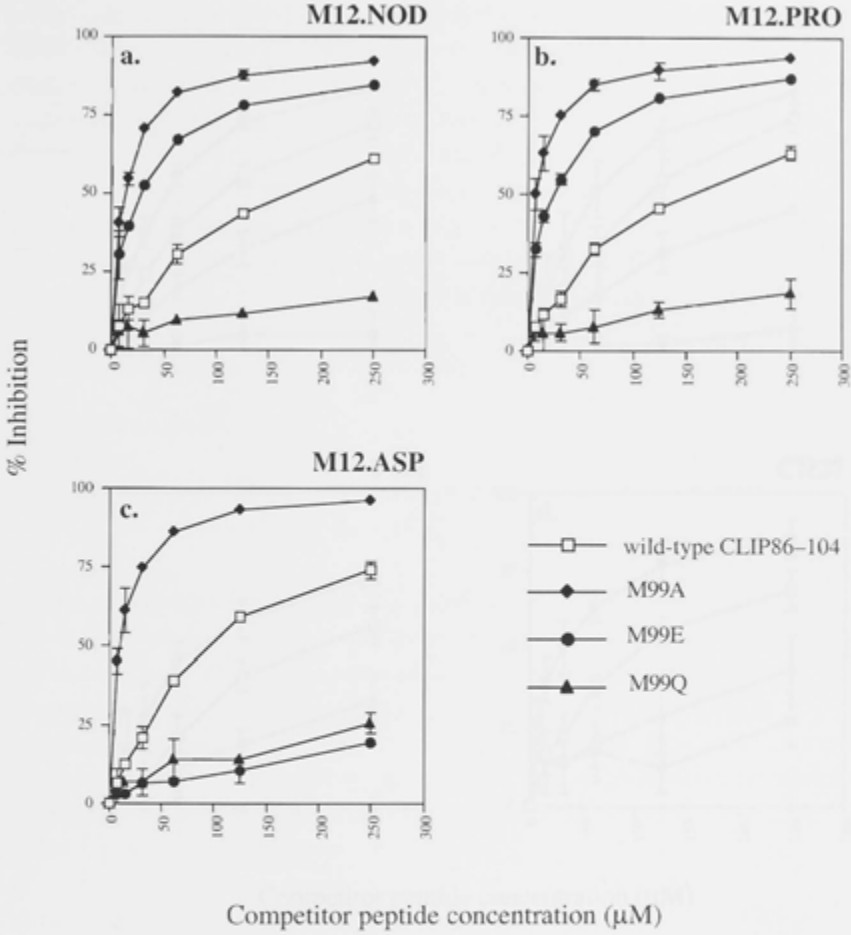


Figure 6.10 *Competition by substituted CLIP analogues for cell-surface binding to mutated I-A^{g7} class II MHC molecules.*

CLIP86–104 analogues substituted at position 99 were examined for their ability to inhibit the cell-surface binding of biotinylated CLIP86–104 (50 μM) to the M12.A3-derived cell lines, M12.PRO (I-A^{g7Pro56}) and M12.ASP (I-A^{g7Asp57}) which express mutated forms of the I-A^{g7} class II MHC molecule. Binding was compared to that obtained with M12.NOD cells, also derived from M12.A3 but which express wild-type I-A^{g7}. Data shown represent the mean percentage inhibition ± SD from six independent experiments. Before calculating % inhibition, median fluorescence intensity (MFI) measurements were normalised for any minor differences in the levels of class II MHC expression between these cell lines, as determined by OX6-FITC staining. Cells were incubated with biotinylated peptide and competitor for 18 hours at 37°C before flow cytometric analysis, as described in *Chapter* 2, §2.7.2.

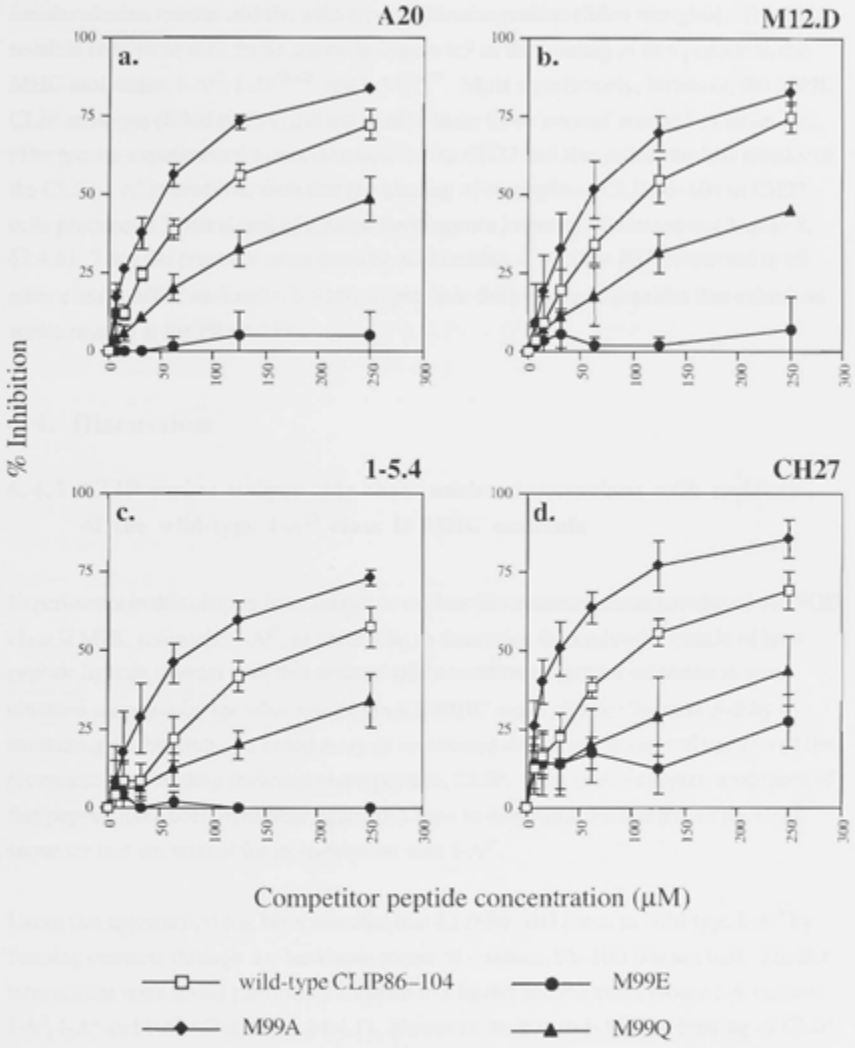


Figure 6.11 *Competition by substituted CLIP analogues for cell-surface binding to diabetes-resistant I-A class II MHC molecules.*

Analogues of CLIP86–104 exhibiting amino acid substitutions at position 99 were examined for their ability to inhibit the cell-surface binding of biotinylated CLIP86–104 to the cell lines, A20 (H-2^d), M12.D (I-A^d), 1-5.4 (I-A^u) and CH27 (H-2^k). Data shown represent the mean percentage inhibition ± SD from two independent experiments. Cells were incubated with biotinylated CLIP (50 μM for A20, M12.D & CH27; 75 μM for 1-5.4) and competitor peptide for 18 hours at 37°C before flow cytometric analysis, as described in *Chapter 2*, §2.7.2.

smaller alanine residue and the wild-type methionine residue (filled triangles). This latter result is consistent with those shown in Figure 6.9 of the binding of this peptide to the MHC molecules, I-A^{g7}, I-A^{g7Pro56} and I-A^{g7Asp57}. Most significantly, however, the M99E CLIP analogue (filled circles) did not bind to these three 'normal' mouse I-A molecules. (The greater experimental errors recorded for the CH27 cell line reflect the low affinity of the CLIP–I-A^{k} interaction, such that the binding of biotinylated CLIP86–104 to CH27 cells produces a lower signal of median fluorescence intensity; discussed in *Chapter 3*, §3.4.6). Thus the presence of an aspartic acid residue at position β57 conserved in all other class II MHC molecules is likely to preclude the binding of peptides that exhibit an acidic residue at the P9 position.

6.4. Discussion

6.4.1 CLIP makes unique side-chain anchor interactions with residues of the wild-type I-A^{g7} class II MHC molecule

Experiments in this chapter have sought to explore biochemical characteristics of the NOD class II MHC molecule, I-A^{g7}, in particular, to determine the molecular details of how peptide ligands interact with this unusual αβ heterodimer. Similar information was obtained successfully for other mouse class II MHC molecules in *Chapters 3–5* by examining the binding of a broad array of substituted and/or length-altered variants of the promiscuously-binding invariant chain peptide, CLIP. Here in this chapter, analogues of this peptide have been used once again, this time to determine the features of the CLIP sequence that are critical for its interaction with I-A^{g7}.

Using this approach, it has been revealed that CLIP86–104 binds to wild-type I-A^{g7} by forming contacts through the backbone atoms of residues, 91–100 (Figure 6.5). Similar interactions were found previously between this ligand and the other mouse I-A variants, I-A^{d}, I-A^{s} and I-A^{k} (*Chapter 4*, §4.4.1). However, unique to I-A^{g7}, the binding of CLIP is also critically reliant upon the formation of additional bonds *via* particular C-terminal residues of the peptide sequence (Figures 6.5 & 6.6, Tables 6.2–6.4). Specifically, the side chains of Leu 97, Arg 100 and Met 102 appeared to form critical anchor interactions with the I-A^{g7} αβ dimer, without which binding was not sustained (Figure 6.6a). Additional positive binding energy was provided through side-chain interactions the residues, Leu 98 and Pro 101, while the native CLIP residues, Pro 96 and Met 99, were inhibitory to binding. The binding to I-A^{g7} of additional longer CLIP ligands (Figure 6.4; Table 6.1), frameshifted 15-mers (Table 6.2), truncated CLIP (Table 6.3) or analogues

that exhibited a combination of L-alanine substitutions and/or truncations (Table 6.4) also differed from that observed previously between these ligands and other mouse class II MHC molecules of both I-A and I-E isotypes (*Chapter 3*, Figure 3.1; *Chapter 4*, Figure 4.2, Tables 4.2–4.4 & *Chapter 5*, Tables 5.1–5.3). For example, while the affinity of CLIP for I-A^d, I-A^k, I-A^u and I-E^d molecules was influenced by changes at the N-terminus, the interaction of this ligand with I-A^{g7} was critically dependent upon the presence of the C-terminal residues, Pro 101 and Met 102. Lastly, a CLIP analogue that exhibited a glutamic acid residue at position 99 instead of the native methionine residue was found to bind with high affinity to the wild-type I-A^{g7} molecule (Figures 6.9 & 6.10). This result is consistent with reports that this $\alpha\beta$ dimer favours ligands that have an acidic residue at relative position, P9 (Reich *et al.*, 1994; Amor *et al.*, 1996; Harrison *et al.*, 1997; Reizis *et al.*, 1997b; Zechel *et al.*, 1997).

In order to interpret the experimental findings reported herein with I-A^{g7}, it is necessary to have an understanding of how peptides typically bind to this class II MHC molecule. To date, a number of research groups have explored the interaction of I-A^{g7} with different antigen-derived peptides and have proposed generalised motifs of the features preferred for binding to this atypical class II MHC variant. Moreover, although an X-ray crystallographic structure of I-A^{g7} has yet to be published, an approximation of the overall three-dimensional molecular arrangement of the atoms comprising its peptide-binding groove may be obtained from the X-ray crystallographic structures of the closely-related mouse class II MHC molecules, I-A^d (Scott *et al.*, 1998) and I-A^k (Fremont *et al.*, 1998b). Indeed, the primary sequences of the α_1 domains of I-A^d and I-A^{g7} are identical (Acha-Orbea & McDevitt, 1987) while there are just 12 amino acid differences between the α_1 domains of I-A^{g7} and I-A^k, (Benoist *et al.*, 1983a). Similarly, the β_1 domain of I-A^{g7} differs from that of I-A^k also by 12 residues and from I-A^d by 17 residues (Acha-Orbea & McDevitt, 1987).

From an examination of such data, it would appear that interactions between antigen-derived peptide ligands and I-A^{g7} are not significantly different from those with other class II MHC molecules. For example, substitution and truncation analyses of a range of synthetic ligands (Reich *et al.*, 1994; Amor *et al.*, 1996; Harrison *et al.*, 1997; Reizis *et al.*, 1997b), together with sequence data of naturally-processed peptides eluted from affinity-purified I-A^{g7} (Reich *et al.*, 1994), have revealed that important contacts are made with I-A^{g7} *via* the amino-acid side chains at the relative positions, P1, P4, P6, P7 and P9 of the bound peptide (Reizis *et al.*, 1997b). By extrapolation from the I-A^d and I-A^k crystal structures (Fremont *et al.*, 1998b; Scott *et al.*, 1998), the positioning of these interactions corresponds, as expected, with the major specificity-determining pockets

within the peptide-binding groove. This has also been borne out in computer-generated homology models of the I-A^{g7} protein structure (Amor *et al.*, 1996; Reizis *et al.*, 1997b) based upon the X-ray crystallographic coordinates of HLA-DR1 complexed with the influenza haemagglutinin peptide, HA306–318 (Stern *et al.*, 1994). Moreover, a ligand bound within the groove of the I-A^{g7} molecule may be predicted to exhibit a similar conformation to that seen in other peptide–class II MHC complexes, since the asparagine residues that are important for forming the main-chain hydrogen-bond network with the ligand in these molecules are also conserved in the I-A^{g7} αβ dimer (positions α62, α69 and β82; *Chapter 4*, §4.4.2; Acha-Orbea & McDevitt, 1987; Liu *et al.*, 1993). Thus, CLIP may be envisaged to bind to wild-type I-A^{g7} with its backbone tethered by an elaborate system of hydrogen bonds, while its downward-oriented side chains are accommodated within the pockets of the peptide-binding groove.

6.4.2 Most of the favourable interactions between CLIP side chains and wild-type I-A^{g7} do not take place at conventional anchor pockets

The participation of both backbone and side-chain atoms in the positive binding interactions of CLIP with the wild-type I-A^{g7} αβ dimer is unusual for a molecule of the I-A isotype (Figure 6.6a). Analysis of CLIP binding to I-A^{d}, I-A^{u} and I-A^{k} identified few, if any, favourable side-chain contacts (*Chapter 3*, Figures 3.2, 3.3 & 3.6). In order to understand specifically how the C-terminal CLIP residues, Leu 97, Leu 98 and Arg 100–Met 102 might make favourable contacts with I-A^{g7}, it is necessary to consider the probable spatial arrangement of the atoms of this αβ dimer in the vicinity of the predicted interaction sites. Such information is collated in Table 6.6 for the peptide-binding groove of I-A^{g7} and is based upon data from homology models of this molecule (Amor *et al.*, 1996; Reizis *et al.*, 1997b), X-ray crystallographic studies of other class II MHC variants (Stern *et al.*, 1994; Ghosh *et al.*, 1995; Fremont *et al.*, 1998b; Scott *et al.*, 1998), sequence comparisons (Acha-Orbea & McDevitt, 1987; Davis *et al.*, 1989; Liu *et al.*, 1993) and analyses of a subset of peptide ligands that bind to I-A^{g7} (Reich *et al.*, 1994; Amor *et al.*, 1996; Harrison *et al.*, 1997; Reizis *et al.*, 1997b, 1998). For comparison, data is also shown in Table 6.6 for the corresponding interactions of CLIP side chains with the pockets of the closely-related I-A^{d} and I-A^{k} class II MHC molecules, as detailed previously in *Chapter 3*, Table 3.2.

Firstly, it is significant to note that the unusual anchor interactions between CLIP and I-A^{g7} do not appear to arise from a change in the frame of binding. Indeed, the demonstration that the side chain at position Ii99 of the CLIP sequence interacts with the

Table 6.6 *The interaction of CLIP side chains with the pockets of the I-A^{g7} peptide-binding groove.*

Shown are the amino acid residues whose atoms contribute to the major polymorphic pockets of the peptide-binding groove of I-A^{g7}. This information is presented in comparison to that of the closely-related class II MHC molecules, I-A^{d} and I-A^{k}, shown previously in *Chapter 3*, Table 3.2. For I-A^{g7}, residues have been taken from homology models of this αβ dimer, where possible (Amor *et al.*, 1996; Reizis *et al.*, 1997). Alternatively, residues have been extrapolated from equivalent analyses and X-ray crystallographic studies of I-A^{d} and I-A^{k} (Lee & McConnell, 1995; Tate *et al.*, 1995; Liang *et al.*, 1996; Nelson *et al.*, 1996; Bartnes *et al.*, 1997; Fremont *et al.*, 1998b; Nydam *et al.*, 1998; Scott *et al.*, 1998; Weber *et al.*, 1998). Within each pocket, these residues form either hydrogen bonds or van der Waals interactions with the atoms of the amino acid side chains of a bound peptide. Throughout this thesis, class II MHC residues are numbered according to the secondary structure-based sequence alignment of the α_1 and β_1 domains of the molecules, I-A^{k}, I-E^{k} and HLA-DR, reported by Fremont *et al.* (1998b). The primary amino acid sequences of the α_1 and β_1 domains of the different I-A molecules are taken from Acha-Orbea & McDevitt (1987), Davis *et al.* (1989) and Liu *et al.* (1993). Polymorphic residues between the I-A molecules are italicised. Those underlined confer a specific physicochemical property to the pocket to govern its overall peptide side-chain preference. For I-A^{g7}, the peptide side chains commonly found at each position have been taken from alignments of naturally-processed ligands of this molecule (Reich *et al.*, 1994; Amor *et al.*, 1996; Harrison *et al.*, 1997; Reizis *et al.*, 1997, 1998). The individual side chains of the native CLIP sequence that bind at these positions are shown in italics beneath the respective pocket number.

[a]The nature of the interaction of the native CLIP side chains is summarised from the cell-surface binding assay (pH 7.0; Figure 6.6) presented in this chapter.

[b]βAsp 57 is mutated to a serine residue in the I-A^{g7} class II MHC molecule from the non-obese diabetic (NOD) mouse. The presence of a non-Asp residue at this position has been linked to the development of autoimmune diabetes in both these animals and in humans.

⚓ represents an anchor side chain within the CLIP sequence, such that binding to the I-A molecule is not sustained if this residue is substituted with L-alanine.

✓ represents the provision of positive binding energy by this CLIP side chain at the binding interface.

↯ represents the provision of negative binding energy by this CLIP side chain at the binding interface.

— represents no notable contribution of binding energy by the CLIP side chain to the interaction with I-A or, in the case of the wild-type CLIP residue at the P4 position, Ala 94, where this side chain was not examined by substitution analysis.

Allotype	P1 *Met 91*	P4 *Ala 94*	P6 *Pro 96*	P7 *Leu 97*	P9 *Met 99*
I-A^{g7}	*αHis 24*, αPhe 54, βAsn 82, *βThr 85*	*αAsn 62*, *βPhe 11*, βThr 71, βGlu 74	*αThr 11*, *αAsn 62*, *αGlu 66*, *βHis 9*, *βPhe 11*	*αAsn 69*, βTyr 30, *βTyr 47*, *βTyr 61*	*αAsn 69*, *αIle 72*, *αLeu 73*, αArg 76, *βSer 57*^{b}, *βTyr 60*, *βTyr 61*
Pocket character:	large, unrestricted	medium, amphiphilic	small, polar	large, amphiphilic	medium, basic
Residues observed:	*e.g.* Leu, Tyr, Glu, Gln	*e.g.* Leu, Ile, Val	*e.g.* Ala, Thr, Ser, Val	e.g. Leu, Arg, Tyr, Asn	*e.g.* Glu, Asp
[a]CLIP interaction:	—	—	↙	⚓	↙
I-A^{d}	*αHis 24*, *αLeu 31*, αPhe 32, αTrp 43, *αIle 52*, *αLeu 53*, αPhe 54, βAsn 82, *βPro 85*	αTyr 9, αGly 9a, *αAsn 62*, *βPhe 11*, *βGly 13*, *βThr 28*, βGlu 74, *βAla 78*	*αAsn 62*, *αAla 65*, *αGlu 66*, *βVal 9*, *βPhe 11*, βTyr 30	*αAsn 69*, *βThr 28*, βTyr 30, *βTrp 61*, *βIle 67*, βThr 71	*αHis 68*, *αAsn 69*, *αIle 72*, αArg 76, *βVal 38*, *βAsp 57*^{b}, βTyr 60, *βTrp 61*
Pocket character:	large, unrestricted	medium, hydrophobic	shallow, amphiphilic	shallow, hydrophobic	medium
Residues observed:	*e.g.* Tyr, Thr, Glu, Ser	*e.g.* Val, Ile, Leu, Ala	*e.g.* Ala, Val, Ser	*e.g.* Ala, Leu, Gly, Val	*e.g.* Ala, Ser, Val
[a]CLIP interaction:	—	—	↙	—	↙
I-A^{k}	*αPhe 24*, *αLeu 31*, αPhe 32, αTrp 43, *αArg 52*, *αArg 53*, αPhe 54, βAsn 82, *βThr 85*	αTyr 9, αGly 9a, *αAsn 62*, *βPhe 11*, *βPro 13*, *βLeu 26*, *βIle 28*, βGlu 74, *βVal 78*	*αThr 11*, *αAsn 62*, *αThr 65*, *αGly 66*, *αAsn 69*, *βHis 9*, *βPhe 11*, βTyr 30	*αThr 65*, *αAsn 69*, *βIle 28*, βTyr 30, *βTyr 47*, *βTrp 61*, βGln 64, *βTyr 67*, *βArg 70*	*αHis 68*, *αAsn 69*, *αIle 72*, *αLeu 73*, αArg 76, *βTyr 37*, *βAsp 57*^{b}, *βTrp 61*
Pocket character:	medium, amphiphilic	large, hydrophobic	medium, polar	shallow, hydrophobic	medium, amphiphilic
Residues observed:	*e.g.* Asp, Glu, Thr, Cys	*e.g.* Ile, Val, Leu, Asn	*e.g.* Glu, Gln	no basic residues	*e.g.* Ser, Thr, Ala, Gly
[a]CLIP interaction:	✓	—	↙	—	—

P9 pocket of I-A^{g7} (Figure 6.9) indicates that this ligand binds within the groove of this heterodimer in the same alignment as determined previously for the binding of CLIP to HLA-DR (Ghosh *et al.*, 1995), I-A (*Chapter 3*, §3.4.4; Lee & McConnell, 1995) and I-E molecules (*Chapter 5*, §5.4.2). Thus, the first pocket in the I-A^{g7} peptide-binding groove, P1, accommodates the CLIP side chain, Met 91. The lack of effect observed when this residue was substituted for the smaller alanine in Figure 6.6a is consistent with a previous consensus motif reported for peptides binding to I-A^{g7} suggesting that this pocket is particularly degenerate (Reizis *et al.*, 1997b). Indeed, these authors tested a number of amino acids at P1 in a synthetic polyalanine nonamer that was anchored only at relative positions, P7 and P8, and found that representatives of all the major classes of amino acid side chains were accommodated efficiently. A similar lack of a specific side chain preference has been reported for the P1 pocket of I-A^{d} (Bartnes *et al.*, 1997; For a full description of the P1 pocket of I-A^{d}, see *Chapter 3*, §3.4.4). Amor *et al.* (1996) have shown from their homology model of I-A^{g7} complexed with the peptide, PLP56–70, from mouse proteolipid protein, that the side chain of the Asn 62 residue that binds in the P1 pocket may hydrogen bond with βThr 85.

The role of the native CLIP residue that corresponds to the I-A^{g7} P4 pocket was not examined by substitution analysis in this study, being already L-alanine (Ala 94). However, it is likely that this small residue is accommodated passively at this position. Previous peptide-substitution analyses have shown that this site in I-A^{g7} receives aliphatic residues most favourably but aromatic or small polar side chains may also be accepted (Reizis *et al.*, 1997b). Amor *et al.* (1996) have also proposed that there might be a specific bias towards a basic residue at this position. Indeed, such a preference was apparent when these authors aligned the binding frames of the peptides, PLP56–70 and rMOG8–22. However, Reizis *et al.* (1997b) have found that the introduction of a positively-charged side chain at this position into their anchored polyalanine nonamer caused a decrease in binding. In this respect, it is interesting to note that the residue suggested by Amor *et al.* (1996) to confer the preference for a basic residue at P4, βGlu 74, is also conserved in I-A^{d} and I-A^{k} and neither of these molecules has been reported to bind positively-charged residues at this position (Liu *et al.*, 1993). Similarly, I-E^{k} shows a preference for aliphatic or polar P4 side chains despite the presence of βGlu 74, while the I-E^{d} heterodimer does show a clear bias for basic residues at this position yet the β74 position in this molecule is occupied by a serine. Instead, the acidic character of the P4 pocket of I-E^{d} is provided by βAsp 70, a position occupied by an arginine residue in I-A^{g7} (For a full discussion of the peptide-binding motifs of I-E^{d} and I-E^{k}, see *Chapter 5*, §5.4.2). The crystal structures of I-A^{d} and I-A^{k} have shown that the βGlu 74 residue does not confer an acidic character to this pocket but, rather, makes extensive van der

Waals contacts with aliphatic peptide side chain bound at this position (Fremont *et al.*, 1998b; Scott *et al.*, 1998). It is possible that the basic residues observed by Amor *et al.* (1996) to be enriched within I-A^{g7}-binding peptides are instead at the P5 position. Such residues have been noted here in natural ligands of this molecule (Reich *et al.*, 1994). Moreover, the P5 side chain is predicted to be oriented out of the groove (Stern *et al.*, 1994; Ghosh *et al.*, 1995; Dessen *et al.*, 1997; Fremont *et al.*, 1998b; Scott *et al.*, 1998) and basic residues typically represent good TCR contact sites (Alexander *et al.*, 1994).

The next prominent pocket within the peptide-binding groove of I-A^{g7} occurs at relative position, P6. Amino-acid substitution analysis of antigen-derived ligands of I-A^{g7} has revealed that this pocket optimally accommodates small, polar residues (Reizis *et al.*, 1997b). Thus, the enhancement of binding found in this study when the native CLIP residue, Pro 96, was replaced with alanine (Figure 6.6a) is likely to be the result of an alleviation of steric compression at this site. The small molecular dimensions of the P6 pocket are also common to I-A^{d} and I-A^{k} (Fremont *et al.*, 1998b; Scott *et al.*, 1998) by consequence of a large residue at position α66 for I-A^{d} and, similarly, at position β9 for I-A^{k}. In the I-A^{g7} molecule, both of these particular residues are present, most likely making this pocket even smaller. The side chains of αGlu 66 and βHis 9 also contribute to the polar character of this pocket in I-A^{g7}.

The P7 pocket of I-A^{g7} represents the first site at which an anchor interaction takes place *via* a residue of the CLIP sequence, Leu 97 (Figure 6.6a). Substitution analyses of antigen-derived peptides have shown that a broad variety of amino acids may be accommodated here, most likely owing to the large molecular dimensions of this pocket, as revealed by homology modelling of the I-A^{g7} peptide-binding groove (Reizis *et al.*, 1997b). Such degeneracy in the side-chain preferences of the P7 pocket is a feature common to most I-A, I-E and HLA-DR class II MHC molecules (*Chapter 3*, §3.4.4 & *Chapter 5*, §5.4.2). Nevertheless, the side chains that provide the most positive binding energy at this site within I-A^{g7} are large with a hydrophobic component. The Leu 97 residue of the CLIP sequence fulfils both of these requirements. The loss of binding observed in this study when this residue was replaced by alanine (Figure 6.6a), is equivalent to that seen by Reizis *et al.* (1997b) upon a similar substitution within their anchored polyalanine nonamer. For example, the ability of the 9-mer synthetic ligand to inhibit T cell stimulation by antigenic peptide, p12(166–185), increased from just 25% with an alanine residue at P7 to 75% when a leucine residue was introduced.

Lastly, the wild-type CLIP residue at the P9 position, Met 99, is inhibitory to binding (Figure 6.6a) with either an alanine or a glutamic acid residue being more favoured

(Figure 6.9). The unusual specificity of the P9 pocket in I-A^{g7} for an acidic residue and the relationship of this to the I-A^{g7}-specific mutation at position β57 is discussed in detail below (§6.4.3).

Thus, only in the one instance do any of the side-chain anchor interactions shown in this study to be critical for the binding of CLIP to I-A^{g7} occur at conventional specificity-determining pockets within the peptide-binding groove, namely, the residue Leu 97 at P7. The favourable interactions of the side chains, Leu 98, Arg 100, Pro 101 and Met 102 must therefore all take place *via* atypical means. For example, the P8 side chain of ligands bound to I-A^{g7} is predicted to extend outwards from the groove to represent a possible TCR contact site (Amor *et al.*, 1996), an arrangement typical at this position in most class II MHC molecules (Stern *et al.*, 1994; Dessen *et al.*, 1997; Fremont *et al.*, 1998b; Scott *et al.*, 1998). Meanwhile, extrapolating from the crystal structure of CLIP complexed with HLA-DR3, the residues Arg 100 (P10) and Pro 101 (P11) would be expected to lie at the outermost edge of the I-A^{g7} peptide-binding groove, with Met 102 (P12) extending beyond this site altogether (Ghosh *et al.*, 1995).

The formation of these unconventional anchor residues between CLIP and I-A^{g7} is reminiscent of the interaction of CLIP with I-E^{d} molecules reported in *Chapter 5* (Figure 5.8). There too, the residues, Leu 98 and Met 102, together with Thr 95, formed anchor contacts with the class II MHC molecule outside of the pockets of the peptide-binding groove. Similarly, Met 102 was found to provide positive binding energy to the interaction between CLIP and the three I-A allotypes, I-A^{d}, I-A^{k} and I-A^{u} in *Chapter 3* (Figures 3.2, 3.3 &3.6) and Leu 98 was favourable to the association with I-A^{d} (Figures 3.2 & 3.6). As discussed previously, it is difficult to elucidate how these residues contribute positive binding energy, particularly without relevant X-ray crystallographic data. For example, equivalent interactions were not present in the crystal structures of I-A^{d} and I-A^{k} complexed with antigen-derived peptides (Fremont *et al.*, 1998b; Scott *et al.*, 1998) and, therefore, little structural detail of these molecules is available beyond the immediate environment of the prominent specificity pockets. Moreover, the knowledge of how the atoms are arranged around the C-terminus of a peptide bound to I-A^{d} and I-A^{k} may be of limited use anyway with respect to the I-A^{g7} αβ dimer, given the conformational differences that may be present in this area as a result of the βPro56His and βAsp57Ser mutations (discussed subsequently in §6.4.4 & §6.4.5, respectively). Unfortunately, the homology models of I-A^{g7} also fail to provide much information on the structural organisation at the atomic level of this region of the αβ dimer (Amor *et al.*, 1996; Reizis *et al.*, 1997b), as does the crystal structure of CLIP complexed with HLA-DR3 (Ghosh *et al.*, 1995) and homology models of this ligand associated with I-A^{d}, I-A^{u}

and I-A^{k} (Lee & McConnell, 1995). It is interesting to note, however, that the mouse CLIP residues Arg 100, Pro 101 and Met 102 have been identified to be critical for the activation of an I-A^{b}-restricted T cell hybridoma generated in Ii^{-} mice that recognises the peptide, mCLIP86–102 (Naujokas *et al.*, 1998). Similarly, activation of another I-A^{b}-restricted T cell hybridoma, this time recognising human CLIP89–102, is dependent upon the hCLIP residues at positions 100 (Gln) and 101 (Ala) (Morkowski *et al.*, 1995). The availability of these residues as important TCR contact sites when CLIP binds to I-A^{b} is consistent with their predicted exposure to the solvent.

6.4.3 An acidic residue at the P9 position of an I-A^{g7}-binding peptide provides a surrogate charge interaction with αArg 76

In this study, the introduction of a glutamic acid into the CLIP sequence at the P9 position was found to result in a marked increase in the affinity of this peptide for I-A^{g7} (M99E; Figures 6.9 & 6.10). This finding is consistent with previous reports of the P9 pocket of the I-A^{g7} αβ dimer having a preference for acidic residues. For example, such residues are commonly found within the C-terminus of naturally-processed peptides eluted from these molecules (Reich *et al.*, 1994) and are also noted frequently in this region when sequences of synthetic I-A^{g7}-binding peptides are aligned (Amor *et al.*, 1996; Harrison *et al.*, 1997; Zechel *et al.*, 1997). Moreover, Reizis *et al.* (1997b) have found that a glutamic residue at the P9 position in their anchored polyalanine nonamer yielded nearly 100% inhibition of I-A^{g7}-restricted T cell proliferation stimulated by their reference peptide, compared with less than 25% inhibition provided by most other physicochemical types of amino acid side chain tested at this position. Similarly, when the P9 acidic residue is truncated from the C-terminus of a natural I-A^{g7} ligand, such as MSA560–574, binding is impaired severely (Reich *et al.*, 1994).

The ability of an acidic residue to provide positive binding energy to the interaction with I-A^{g7} is considered to be a consequence of the βAsp57Ser mutation in this molecule. Specifically, the inability to form the β57–α76 salt bridge at the base of the P9 pocket leaves the positively-charged αArg 76 residue unpaired and seeking to create a surrogate ion-pairing with a suitable side chain in the peptide. Consistent with this, molecular models of the I-A^{g7} peptide-binding groove appear to show that the side chain of the αArg 76 residue is oriented into the P9 pocket, contributing a basic character to this site and ideally placed to form an ionic interaction with the incoming side chain of the P9 residue of the bound peptide (Amor *et al.*, 1996; Reizis *et al.*, 1997b).

In common with I-A^{g7}, other class II MHC molecules that lack an aspartic acid residue at position β57 also exhibit a preference for negatively-charged side chains at the P9 position of peptide ligands, dictated by the unpaired positive charge of the αArg 76 residue. For example, substitution and truncation analyses of synthetic peptides which bind to HLA-DQ8 (DQA1*0301/DQB1*0302; βAla 57) have revealed that the presence of an acidic residue at P9 yields a ligand capable of binding with high affinity to this molecule (Kwok *et al.*, 1996a, 1996b; Godkin *et al.*, 1997; Oiso *et al.*, 1997). Moreover, such a residue is featured in all predominant naturally-processed peptides eluted from DQ8 (Chicz *et al.*, 1994) and the enrichment of these residues in the Edman degradation cycles 12–14 by pool sequencing confirms their location to be at P9. (Godkin *et al.*, 1997). Peptides exhibiting acidic residues at the P9 position are similarly favoured for binding to HLA-DR4Dw15 (DRA/DRB1*0405; βSer 57), as determined by sequencing individual (Kinouchi *et al.*, 1994) and pooled natural ligands (Friede *et al.*, 1996), together with a binding analysis of substituted peptides (Marshall *et al.*, 1994). Likewise, the determination of peptide-binding motifs for the Lewis rat RT1.B^{l} class II MHC molecule (βSer 57) reveals that this heterodimer preferentially binds peptides with negatively-charged residues at P9 (Reizis *et al.*, 1996; Wauben *et al.*, 1997). Moreover, Reizis *et al.* (1996) have substituted such a residue into mouse CLIP86–102 and observed a 10-fold improvement in the binding of this peptide to RT1.B^{l}, akin to the experimental findings in this study with I-A^{g7} (Figure 6.9 & 6.10).

Although an acidic residue at P9 provides significant positive binding energy to the interaction of peptides with I-A^{g7} through charge pairing with αArg 76, peptides lacking such a residue may still bind to this molecule. This is demonstrated clearly in this study by the ability to measure the binding of both wild-type CLIP and the M99A analogue to I-A^{g7} (*e.g.* Figures 6.9 & 6.10). Harrison *et al.* (1997) have determined similarly that a C-terminal acidic residue is not necessary for peptides to be able to bind to I-A^{g7}. However, close scrutiny of the I-A^{g7} binding motif proposed by these authors reveals a likely error in the alignment of the peptide residues relative to the pockets of the I-A^{g7} groove. For example, the side-chain preferences determined by Harrison *et al.* (1997) for what they term the "P6" and "P9" primary anchors — large, hydrophobic residues and aromatic, hydrophobic or basic residues, respectively — would appear to be better aligned with the P4 and P7 pockets, respectively (Table 6.6). Not only do these side chain preferences fit much better at these positions with other assessments of the binding motif of I-A^{g7} and the molecular model of the I-A^{g7} binding groove constructed by Reizis *et al.* (1997b; described above, §6.4.2) but the interaction of a basic residue at the P9 pocket, as proposed by Harrison *et al.* (1997), is likely to be extremely unfavourable (see below). Instead, the decrease in binding that is observed when a basic residue is substituted into

position "P11" of the HEL10–22 peptide in the study of Harrison *et al.* (1997), suggests that this is the true P9 residue. Moreover, the increase in binding affinity observed when Asn 19 at "P8" was substituted with alanine is more compatible with this residue interacting with the small P6 pocket of I-A^{g7} (Table 6.6). Indeed, the true P8 residue would be unlikely to show such an effect given that it is predicted be oriented predominantly out of the peptide-binding groove to be an important TCR contact site (Amor *et al.*, 1996). The Arg 21 residue at position "P11" fits this role much better, not only because basic residues are frequently critical for TCR recognition (Alexander *et al.*, 1994) but also because the substitution of this residue repeatedly shows a greater disruption of T cell activation than actual binding to the I-A^{g7} molecule (Harrison *et al.*, 1997). Cohen (1997a) and Reizis *et al.* (1998) have suggested similarly that an error exists in the alignment of the peptide-binding motif of Harrison *et al.* (1997) and the alignment of the HEL10–23 peptide shown in Figure 6.8a has been adjusted accordingly. Nevertheless, true to the conclusion of Harrison *et al.* (1997) that peptides lacking acidic P9 residues may still bind to I-A^{g7}, the new alignment of their peptide-binding motif places an uncharged residue, Gly 22, at the P9 position of the HEL10–22 peptide. Furthermore, truncation and substitution analysis of the binding to I-A^{g7} of this peptide did not indicate much significance in the nature of the residue found at this position. Confirmation of the binding of HEL10–23 to I-A^{g7}, despite its lack of an acidic P9 residue, is shown in this study in Figure 6.8. It is worth noting, however, that the substitution of an acidic residue into P9 did create a HEL analogue that bound appreciably well to I-A^{g7} (Harrison *et al.*, 1997). The ability of peptides to bind to this class II MHC molecule without fulfilment of the major anchor requirement at the P9 position is discussed subsequently in §6.4.4.

Just as peptides lacking an acidic P9 residue may still bind to I-A^{g7}, the absence of an aspartic acid residue at position β57 is not an absolute indicator that peptides exhibiting a negatively-charged P9 residues will be the most preferred for binding. HLA-DQ2 (DQA1*0501/DQB1*0201), for example, exhibits an alanine amino acid at position β57 but examination of the peptide sequence features preferred for binding to this heterodimer indicates that there is no specific selection of peptides with negatively-charged residues at P9. Indeed, the predominant naturally-processed ligands to be eluted from this class II MHC molecule exhibit residues with bulky aromatic or hydrophobic aliphatic side chains at the P9 position (Vartdal *et al.*, 1996; van de Wal *et al.*, 1997). Moreover, the results of amino acid substitution analyses of DQ2-binding peptides have indicated that these residues are the most favoured at this position, more so than either glutamic acid or aspartic acid (Johansen *et al.*, 1996; van de Wal *et al.*, 1996; Quarsten *et al.*, 1998). Homology modelling of the HLA-DQ2 αβ dimer based upon the X-ray crystallographic

structure of the HA–HLA-DR1 complex (Stern *et al.*, 1994) has indicated that the substitution of the aspartic acid residue at position β57 by alanine in this molecule causes a change in the dimensions of the P9 pocket from a recessed shelf to a particularly broad and deep cleft (Vartdal *et al.*, 1996; van de Wal *et al.*, 1997; Quarsten *et al.*, 1998). This serves to explain the ability of the HLA-DQ2 molecule to accommodate bulky residues over the small P9 side chains preferred by the mutant DQ2 heterodimer, Mut57-DQ2 (βAsp 57; Quarsten *et al.*, 1998). This latter form has now been identified to be a naturally-occurring allelic variant, designated DQA1*0501/DQB1*0203 (Olerup *et al.*, 1997). Meanwhile, the lack of any strong preference for an acidic P9 residue to interact at this site may be explained by the observation that the free basic moiety of the αArg 76 residue is oriented away from the pocket in the HLA-DQ2 heterodimer and is more ideally located to make interactions with the peptide backbone carbonyl groups than the side chain at position P9 (Quarsten *et al.*, 1998).

The P9 pocket of the I-A^{g7} molecule differs from that of HLA-DQ2, not just by the degree of basicity bestowed upon it by the unpaired αArg 76 residue, but also in terms of its molecular dimensions. Specifically, extrapolation from the crystal structures of the closely-related I-A^{d} (Scott *et al.*, 1998) and I-A^{k} αβ dimers (Fremont *et al.*, 1998b), together with homology modelling of the I-A^{g7} peptide-binding groove (Amor *et al.*, 1996; Reizis *et al.*, 1997b) predicts that this site is relatively small despite the replacement of the βAsp 57 residue for the smaller side chain of serine (Table 6.6). Consequently, I-A^{g7} would be expected to be unable to accommodate bulky side chains at this site. This is demonstrated in this study by the high affinity binding of the M99A peptide to I-A^{g7} compared with that of wild-type CLIP (Figures 6.6, 6.9 & 6.10). Indeed, an alanine residue at this position in the CLIP86–104 sequence was even able to overcome the extremely unfavourable placement of a glutamic acid residue at position 100 (M99A/R100E; Figure 6.9). The ability of the M99A peptide to bind with higher affinity than wild-type CLIP to the other I-A allotypes, I-A^{d}, I-A^{u} and I-A^{k} also confirms the similarly small dimensions of the P9 pocket between these different molecules and I-A^{g7} (Figure 6.11, *Chapter 3*, Figures 3.2 & 3.6; Gautam *et al.*, 1995). Moreover, the resemblance of this outcome with the results obtained with the revertant I-A^{g7Pro56} and I-A^{g7Asp57} molecules indicates that the novel β-chain mutations in wild-type I-A^{g7} do not change significantly the dimensions of this site. As described above, however, they do change the physicochemical characteristics such that I-A^{g7} may bind a peptide with a bulky residue at the P9 position if this residue also exhibits a negative charge to provide sufficient binding energy to compensate for the steric conflict. This is illustrated in this study by comparing the high-affinity binding of the M99E peptide *versus* that of M99Q (Figure 6.10).

As shown in Figures 6.7, 6.9 & 6.10, the reintroduction into I-A^{g7} of an aspartic acid residue at position β57 (I-A^{g7Asp57}) eliminated the ability of an acidic peptide side chain to interact favourably within the environment of the P9 pocket. This result is consistent with the preference for such a residue being related to the unpaired electrostatic charge of the αArg 76 residue since, in these altered I-A^{g7} molecules, the β57–α76 salt bridge is now assumed to form. However, the low affinity of the CLIP M99E, MSA560–574 and p12(166–195) peptides for binding to I-A^{g7Asp57} (Figures 6.7, 6.9 & 6.10) indicates that an acidic residue at this position is now a source of considerable negative binding energy. Similarly, a random 15-mer peptide exhibiting a negatively-charged residue at the P9 position has been shown to bind poorly to the I-A^{g7} double mutant form, I-A^{g7Pro56Asp57} (Oiso *et al.*, 1998). Collectively, these findings suggest that a repulsive interaction may occur between the negative charge of this side chain and that of the restored βAsp 57 residue. Alternatively, this site may generally be too small or too hydrophobic to accommodate a negatively-charged side chain and the water molecules of its associated hydration shell. Indeed, both the restricted volume and hydrophobicity of the P9 pocket have been noted previously in the X-ray crystallographic analyses of HLA-DR1 (Stern *et al.*, 1994), HLA-DR4 (Dessen *et al.*, 1997) and I-A^{d} (Scott *et al.*, 1998). By contrast, the lack of an acidic residue at P9 in the HEL10–23 peptide (Gly 22 at this site) allows this ligand to bind unhindered to I-A^{g7Asp57}. Interestingly, however, the rMOG8–22 peptide also binds freely to the I-A^{g7Asp57} αβ dimer, despite exhibiting an acidic P9 residue in the I-A^{g7} binding frame of this peptide, as determined by Amor *et al.* (1996). In this respect, it is possible that this peptide may exhibit two separate epitopes in different registers. This is not unheard of, indeed, overlapping epitopes within a peptide have even been described for a single class II MHC molecule (Zamvil *et al.*, 1986; Quaratino *et al.*, 1995) and may even exhibit similar antigenic surfaces to be recognised by the same T cell clone (Quaratino *et al.*, 1995). The binding frame of the rMOG peptide for interaction with the I-A^{g7Asp57} molecules may be just one amino acid different from that proposed by Amor *et al.* (1996) whereby Gly 9 represents the P1 residue, likely to be amply accommodated in the corresponding unrestricted pocket (Table 6.6). This would then align Ile 12 with the P4 pocket, which indeed prefers aliphatic residues, Ala 14 with the small P6 pocket, Leu 15 with the large P7 pocket, again a highly favoured residue at this position and, lastly, the unobtrusive Gly 17 at the P9 pocket, thereby avoiding both steric and electrostatic conflicts at this critical position.

The unfavourable nature of the interaction between a negatively-charged side chain and the P9 pocket of the I-A^{g7Asp57} αβ dimer suggests that this may be the case also for other class II MHC molecules in which the β57–α76 salt bridge is intact. Consistent with this

notion, the M99E CLIP analogue was found to be unable to compete for binding to such molecules on the surface of the cells, A20 (H-2^d), 1-5.4 (I-A^s) and CH27 (H-2^k) in this study (Figure 6.11). Nevertheless, it was feasible that the inability of ligand to bind was related simply to steric strain provided by the bulky side chain of the Glu 99 residue within the small P9 pocket of these molecules (*Chapter 3*, Table 3.2). This possibility was excluded by the finding that a glutamine residue at P9 did support some binding of CLIP, *i.e.* an uncharged residue of similar shape and size (M99Q; Figure 6.11). Reizis *et al.* (1997b) have also observed a reduction in the binding of CLIP to I-A^d by the introduction of glutamic acid into the P9 position. Moreover, a number of other reports exist in the literature noting the inability of different class II MHC molecules that exhibit βAsp 57 to accommodate a negatively-charged residue at the P9 position, as determined by testing amino acid-substituted peptides. For example, the 34ps peptide, derived from GAD 65 (residues 253–263), has a glutamic acid residue at the P9 position (Glu 261) and binds with high affinity to HLA-DQ8 but not to HLA-DQ3.1 (DQA1*0301/DQB1*0301; Kwok *et al.*, 1996a). These proteins differ by just 6 amino acids, including residue β57 (Ala in DQ8; Asp in DQ3.1). Furthermore, the 34ps peptide does not bind to HLA-DQ3.3 (DQA1*0301/DQB1*0303) which is identical in sequence to HLA-DQ8 except at position β57 (Asp in DQ3.3). Binding of 34ps to HLA-DQ3.3 may be induced however, by the substitution of Glu 261 for an alanine residue, although this peptide now no longer readily binds to DQ8. Further examples of the inability of negatively-charged residues to be accommodated at the P9 position of class II MHC molecules exhibiting βAsp 57 have been detailed by Sette *et al.* (1993), Marshall *et al.* (1994), Kwok *et al.* (1996b) and Oiso *et al.* (1997). This phenomenon is also indicated by the extreme infrequency of finding acidic residues in the P9 position in the predominant natural ligands eluted from different human and mouse class II MHC molecules that express βAsp 57, as apparent in peptide elution and sequencing studies of Falk *et al.* (1994), Friede *et al.* (1996) and Rammensee *et al.* (1995).

It has also been noted that peptides that exhibit basic residues at the P9 position are incapable of binding to I-A^{g7} (Reich *et al.*, 1994; Katoh *et al.*, 1996; Reizis *et al.*, 1997b). Similarly, peptides of this type are extremely disfavoured for binding to other class II MHC molecules that lack an aspartic acid at the β57 position, *e.g.* HLA-DQ8 (Kwok *et al.*, 1996a) and RT1.B^l (Reizis *et al.*, 1996; Wauben *et al.*, 1997). This observation has led to the proposal that the inability of these peptides to bind to these molecules is the result of a repulsive interaction with the unpaired positive charge of the αArg 76 residue. Consistent with this, site-directed mutagenesis of the βAsp 57 residue to serine in the HLA-DR molecule, DRA/DRB1*1301, caused a significant decrease in the ability of this molecule to bind the peptide, MBP152–170, which exhibits an arginine at position P9

(Hurley & Steiner, 1995). Furthermore, peptides with basic residues at the P9 position may bind to other class II MHC molecules in which the β57–α76 salt bridge is intact, *e.g.* HLA-DQ7 (DQA1*0301/DQB1*0301; Godkin *et al.*, 1997), DQ1 molecules with the gene product of the β-chain allele, *DQB1*05032* (Wucherpfennig & Strominger, 1995), HLA-DR2a (DRA/DRB5*0101; Marshall *et al.*, 1994) and all molecules of the I-E isotype (Rammensee *et al.*, 1995; Schild *et al.*, 1995). Nevertheless, for these latter two cases at least, it is known that basic residues at this position are favoured for binding specifically due to acidic character bestowed upon the P9 pocket by the residues βAsp 30/ βAsp 37 and βGlu 9, respectively (For a full description of the physicochemical characteristics of the I-E P9 pocket, see *Chapter 5*, §5.4.2). Instead, it would appear that the inability to bind peptides exhibiting basic residues at the P9 position is not a feature unique to class II MHC molecules that lack an aspartic acid at position β57. For example, it has been observed that such peptides will also not bind to I-A^{d} (Bartnes *et al.*, 1997; Reizis *et al.*, 1997b). Nor are they readily bound by HLA-DR4Dw4 (DRA/DRB1*0401; Sette *et al.*, 1993), HLA-DQ3.1 and DQ3.3 (Kwok *et al.*, 1996a) or even the double revertant, I-A^{g7Pro56Asp57} (Oiso *et al.*, 1998), all of which exhibit an intact β57–α76 salt bridge. Moreover, peptides with basic P9 residues bind poorly to both HLA-DQ2 and its βAsp 57 mutant form, Mut57-DQ2 (Johansen *et al.*, 1996; van de Wal *et al.*, 1996, 1997; Quarsten *et al.*, 1998) despite much of the potential basicity of the DQ2 P9 pocket having been deflected away from this site, as described above (Quarsten *et al.*, 1998). As with the inability of acidic residues to be accommodated at the P9 pocket of most class II MHC molecules, side chains that exhibit positive charges may be simply disfavoured at this site due to the general size restrictions (*e.g. Chapter 3*, Table 3.2).

6.4.4 The lack of the β57–α76 salt bridge in I-A^{g7} reduces the stability of this class II MHC molecule

The formation of favourable contacts between the I-A^{g7} αβ dimer and the CLIP side chains, Leu 97, Leu 98 and Arg 100–Met 102 is unusual (Figure 6.6). As reported in *Chapter 3* (§3.4.2) and by Gautam *et al.* (1995), L-alanine scanning experiments performed on CLIP binding to other mouse class II MHC molecules of the I-A isotype, I-A^{d}, I-A^{k} and I-A^{s} have shown that in only rarely did any CLIP side chains provide significant positive binding energy to the interaction, *e.g.* Leu 98 with I-A^{d} at pH 5.0 (Figure 3.6). Instead, the majority of residues within the CLIP sequence were either neutral or inhibitory to binding and the association was maintained predominantly by the formation of the hydrogen bond network between atoms of the peptide backbone and conserved residues of the binding groove.

Like the preference of I-A^{g7} molecules to bind peptides with acidic residues at the P9 position (§6.4.3), the anchor interactions between the C-terminal residues of CLIP and this molecule may also be attributed to the absence of an aspartic acid residue at position β57, since all but one of the major favourable interactions (that of Leu 97 in the P7 pocket) were found to disappear when such a residue was reintroduced into I-A^{g7} (Figure 6.6c). As noted previously (§6.1.5), the lack of the βAsp 57 residue in I-A^{g7} is predicted to cause destabilisation in the surrounding region of the heterodimer through the inability to form the β57–α76 salt bridge, a critical link between the α_1 and β_1 domains (*e.g.* Nalefski *et al.*, 1995; Scott *et al.*, 1998; for a description of the domain organisation of class II MHC molecules, see *Chapter 1*, Figure 1.3). Indeed, the disruption of this electrostatic interaction in I-A^{d} using site-directed mutagenesis has been shown to result in a reduction in the numbers of class II MHC molecules that reach the cell surface (Nalefski *et al.*, 1995). This suggests that the molecules tend to be folded incorrectly and, consequently, may be targeted for degradation or simply accumulate intracellularly unable to traffic to the plasma membrane. Even in those dimers that do transport efficiently, the residues of the groove that are normally involved in bonds with peptide ligand may no longer be held in the correct orientation for such interactions to occur. The favourable contacts formed between I-A^{g7} and the C-terminal CLIP side chains may therefore be a means to counteract the greater flexibility of this region of the heterodimer and to tether the C-terminal region of the peptide in place where the formation of conventional bonds may be impaired.

In support of the notion that the binding of CLIP86–104 to I-A^{g7} is reliant upon the formation of stabilising contacts additional to, or stronger than, those observed in other CLIP–I-A interactions, longer CLIP ligands were observed to bind preferentially to this molecule (Figure 6.4a & Table 6.1). Such ligands offer greater potential for the formation of both hydrogen bonds and side-chain interactions. This feature should not be confused with the inability of the truncated CLIP ligands in Table 6.3 to bind to this molecule since this also reflects the disruption of the main-chain hydrogen-bond network conserved in all peptide–class II MHC complexes (*e.g.* see *Chapter 4*, Table 4.2 and *Chapter 5*, Table 5.1). However, in this respect, the I-A^{g7}–CLIP interaction was again more sensitive to these truncations than other class II MHC molecules under the same conditions (pH 7.0), a further indication of the reduced affinity of this molecule with regard to binding peptides. The reduction in the effect of having extra residues on the CLIP86–104 ligand observed with I-A^{g7Asp57} also substantiates the idea that the need for additional interactions arises from the absence of the βAsp 57 residue in wild-type I-A^{g7}. Consistent with this, longer CLIP ligands have been shown to bind better than shorter

variants to HLA-DQ2 molecules, which similarly lack an aspartic acid at position β57 (van de Wal *et al.*, 1996; Vartdal *et al.*, 1996).

As discussed previously (§6.4.3), the presence of an acidic residue at the P9 position of an I-A^{g7} peptide ligand provides a significant measure of positive binding energy to the complex by providing a stabilising interaction to compensate for the loss of the β57–α76 salt bridge. It is probable that the additional bonds formed between the I-A^{g7} heterodimer and CLIP also serve in the same manner to uphold the complex and might represent an alternative to having an acidic residue in the P9 position of the peptide. Indeed, the truncated ligand, CLIP90–100, lacks the potential to form several of the requisite C-terminal anchor interactions and, accordingly, this peptide is unable to bind to the I-A^{g7} class II MHC molecules (Table 6.3; Reizis *et al.*, 1997b). However, the binding of this same peptide may be restored simply by the introduction of a glutamic acid residue at the P9 position (Reizis *et al.*, 1997b; sCLIP89–99 in the mouse Ii numbering used by these authors). Thus, it may be envisaged that all ligands that bind to I-A^{g7} but lack an acidic P9 residue similarly exhibit other means of stabilising their interaction. For example, Harrison *et al.* (1997) found that the identity of the residues at relative positions P4 and P7 in the HEL peptide (using the adjusted alignment described above, §6.4.3) were extremely critical for binding to I-A^{g7}. It is possible that anchor interactions at these positions are necessary for stabilising the complex in the absence of an acidic P9 residue to perform this task. Indeed, these authors do offer the suggestion that an acidic residue in the C-terminal region of the peptide might be an alternative to a non-tolerated residue at one of these positions (Harrison *et al.*, 1997).

The results presented herein are particularly interesting in light of the recent debate regarding whether or not I-A^{g7} class II MHC molecules exhibit a general defect in their ability to form stable complexes with peptide ligands. Providing most support to this claim are the experimental findings of Carrasco-Marin *et al.* (1996). Firstly, these authors have reported experiencing considerable difficulties in being able to demonstrate the association of peptides with affinity-purified I-A^{g7} *in vitro*. I-A^{g7} molecules extracted from APC also showed only weak interactions with the radioactive peptides with which they had been cultured. Next, these authors examined kinetic features of peptide-bound, radiolabelled I-A^{g7} and found that the half-life of these complexes was shorter than that of other peptide-bound I-A variants, *e.g.* 5.5 h for I-A^{g7}, compared with 9.3 h for I-A^{d} and 13.4 h for I-A^{b}. Moreover, they observed that I-A^{g7}-expressing APC pulsed with antigenic peptides rapidly lost the ability to stimulate T cell hybridomas when the cultures were washed free of peptide, a result indicative of short-lived peptide–MHC complexes. By contrast, the levels of IL-2 produced by stimulated I-A^{k}-restricted T cells were

equivalent regardless of whether the APC were washed free of excess HEL46–61 peptide or cultured continuously in its presence. The long-lived nature of HEL–I-A^{k} complexes has been demonstrated previously by Nelson *et al.* (1994). Lastly, Carrasco-Marin *et al.* (1996) noted a correlation between the half-lives of different class II MHC molecules at the cell surface and the ability of these molecules to withstand dissociation of the αβ subunits upon SDS-PAGE, *e.g.* the vast majority of I-A^{g7} heterodimers (99%) dissociated into their constituent monomers while 78% of I-A^{b} molecules migrated as stable dimers.

Regarding firstly the difficulties of Carrasco-Marin *et al.* (1996) to demonstrate peptide binding to I-A^{g7} *in vitro*, it is interesting to note that no such problems were encountered in this study using the flow cytometry-based peptide binding assay to measure the binding of biotinylated CLIP86–104. Indeed, it was easier to detect biotinylated CLIP binding to cell-surface I-A^{g7} molecules than to I-A^{k} and I-A^{s} in *Chapter 3* (*e.g.* compare MFI levels between Figure 3.2 and Figure 6.3) although this may simply reflect differences in the total numbers of class II MHC molecules expressed by the respective APC rather than overall affinity for CLIP. Nevertheless, this result was not restricted to the interaction of CLIP with I-A^{g7} but binding was also able to be demonstrated using this method with the antigen-derived peptides MSA560–574 and p12(166–185) (Figure 6.7) and rMOG8–22 and HEL10–23 (Figure 6.8). Other investigators too have successfully measured peptide binding to I-A^{g7} class II MHC molecules. For example, Harrison *et al.* (1997) conducted their examination of the binding of the peptide HEL10–23 with affinity-purified I-A^{g7} in an ELISA-type assay, while Reizis *et al.* (1997b) used an antigen presentation assay to examine different peptide–I-A^{g7} interactions. In this respect, it is possible that the contrasting findings are simply a consequence of the distinct experimental systems and reagents used by the different investigators.

Secondly, in consideration of the reduced lifespan of peptide–I-A^{g7} complexes claimed by Carrasco-Marin *et al.* (1996), Reizis *et al.* (1997b) have provided conflicting data that shows that metabolically-labelled I-A^{g7} molecules immunoprecipitated from NOD splenocytes exhibit a normal lifespan, comparable to that of I-A^{s} molecules. These authors have also shown that the HSP60 peptide, p12(166–185), binds to I-A^{g7} on the surface of glutaraldehyde-fixed macrophages to form a very stable complex, still able to trigger significant T cell activation 8 days after the washout of free peptide. Moreover, this is not simply the result of the peptide being cross-linked to the MHC αβ dimer, for example, through the side chain of the Lys 180 residue, because greater than 50% of the initial T cell response was still observed after 24 h of dissociation using unfixed splenic APC; parameters deemed similar to those observed with other peptide-bound class II

MHC molecules (Reizis *et al.*, 1997b). However, in this respect, the p12(166–185) peptide used in these experiments is perhaps not truly representative of the majority of I-A^{g7}-binding peptides since the binding of this peptide was also shown by these authors to confer the very rare feature of SDS-stability to I-A^{g7} heterodimers (see below).

The only finding of Carrasco-Marin *et al.* (1996) to be accepted widely to date is that concerning the inability of I-A^{g7} class II MHC dimers to withstand denaturation in the presence of SDS detergent. The agreement on this matter comes in spite of the experimental variation observed between laboratories in the proportional estimates of compact dimers formed (Carrasco-Marin *et al.*, 1996; Reizis *et al.*, 1997b; discussed previously, §6.1.5). Incidentally, it is difficult to determine where the SDS-PAGE data presented in Figure 6.1 of this study fits in, given the absence of any quantitative measurements of the respective bands by laser densitometry. Regardless, the finding that a lower proportion of compact I-A^{g7} αβ heterodimers are formed upon SDS-PAGE than other class II MHC variants has perhaps only complicated our efforts to understand the role of these particular molecules in the development of autoimmune diabetes in NOD mice since the functional significance of this phenomenon, if any, remains unknown. For example, debate continues over whether the ability to withstand SDS-induced dissociation has any relevance on the overall lifespan of the peptide–class II MHC complex. Nelson *et al.* (1994) have reported that SDS-stable I-A^{k} complexes exhibit half-lives up to twice as long as unstable complexes of this same class II MHC variant. However, Reizis *et al.* (1997b) have shown that both the SDS-stable and unstable forms of I-A^{g7} exhibit similar lifespans within the APC. Likewise, SDS-stable and unstable forms of HLA-DQ8 persist in the cell for equivalent lengths of time (Reizis *et al.*, 1997a). It has also been shown that the behaviour of a given peptide–class II MHC complex in SDS is unrelated to whether it may be recognised by CD4^{+} T lymphocytes (Wu *et al.*, 1996).

The HSP60 peptide, p12(166–185), is unique amongst I-A^{g7}-binding ligands in exhibiting the ability to stabilise this dimer in the presence of SDS detergent (Reizis *et al.*, 1997b). Substitution analysis has shown that this capacity is dependent largely upon the identity of the residue present at the relative peptide position, P1 (Reizis *et al.*, 1997a). This finding is consistent with the currently-held belief that SDS-stability in class II MHC molecules relates to the fulfilment of side-chain anchor preferences at the pockets within the peptide-binding groove (discussed previously in *Chapter 3*, §3.4.3 & §3.4.6). The actual significance of these interactions has been proposed to lie in the resultant burial of hydrophobic residues within the class II MHC dimer, these being the primary sites to which the SDS molecules bind (Natarajan *et al.*, 1999b). In simplistic terms, it is envisaged that a class II MHC molecule with a peptide associated only tenuously is likely

to be more loosely folded such that more non-polar surface is exposed. The detergent molecules may then bind in a co-operative manner to these regions, bringing about the loss of the peptide and triggering the dissociation of the α and β subunits.

Consistent with the proposed molecular basis for SDS stability described above, Carrasco-Marin *et al.* (1996) used the finding that I-A^{g7} dimers rarely withstood SDS-PAGE to uphold their argument that these molecules are intrinsically unable to form strong interactions with peptide ligands. However, the concept that peptides must only be associated weakly if they do not confer SDS-stability to the αβ dimer is still disputed. For example, Nelson *et al.* (1996) have shown that the contacts required for the formation of SDS-stable HEL(46–61)–I-A^{k} complexes represent only a subset of those needed to maintain peptide binding. This strongly supports the idea that class II MHC heterodimer stability in SDS detergent is quite a separate property from binding strength. In this respect, it is interesting to note that the p12 analogues that exhibited P1 substitutions and were unable to trigger the formation of SDS-stable I-A^{g7} molecules nevertheless showed equivalent binding capacities to those capable of conferring it, as measured in a competitive binding assay (Reizis *et al.*, 1997a).

Collectively, the data described above indicate that I-A^{g7} molecules do form stable interactions with peptide ligands but only if they fulfil one of two criteria: either an acidic residue at P9 (*e.g.* as shown by the peptides, p12(166–185), MSA560–574, rMOG8–22) or the means elsewhere within the peptide to form strong bonds with the αβ heterodimer. These latter interactions may be in the form of unconventional side chain-dependent contacts, such as those seen in this study with the CLIP ligand or, alternatively, by fulfilling anchor preferences elsewhere in the I-A^{g7} peptide-binding groove, as proposed above for the HEL10–22 peptide (Harrison *et al.*, 1997). Whatever the specific source, these bonds serve to compensate for the loss of the β57–α76 salt bridge and provide much needed stability to the I-A^{g7} peptide-binding groove. In this respect, I-A^{g7} may indeed be considered to exhibit a general defect in peptide binding since these requirements no doubt reduce considerably the subset of ligands that are able bind to this molecule. Indeed, a single βAsp57Ser mutation in I-A^{d} has been shown to cause significant impairment in the ability of these molecules to present their typical ligands (Nalefski *et al.*, 1995). This may also be extrapolated to other class II MHC molecules that similarly lack the βAsp 57 residue. For example, Marshall *et al.* (1994) have shown that the substitution of an aspartic acid at position β57 for a serine in HLA-DR molecules leads to a dramatic decrease in the binding affinity of the class II MHC molecule for a simple polyalanine peptide anchored at relative positions, P1 and P5, *e.g.* DRB1*0401 (βAsp 57), IC_{50} = 13 nM compared with DRB1*0405 (βSer 57), IC_{50} = 14000 nM.

Furthermore, the difference in binding capacity observed between the best and poorest ligands of the HLA-DQ2 molecule was considerably greater than that observed for the Mut57-DQ2 variant (Quarsten *et al.*, 1998). The consequences *in vivo* of these effects on antigen presentation are discussed in §6.4.6.

Meanwhile, the inability of I-A^{g7} to form complexes that are stable under the conditions of SDS-PAGE should only be taken to mean that the binding of peptides does not induce the molecule to adopt the same structural form as when such ligands bind to other class II MHC αβ dimers— one should be wary of using this technique as a means to measure peptide affinity. In this respect, Nabavieh *et al.* (1998) have shown by expressing these molecules in different cellular backgrounds that SDS-instability is a feature intrinsic to I-A^{g7}, one unaffected by the specific antigen-processing and presentation machinery of the NOD environment. Similarly, Peterson & Sant (1998) have shown that this feature is not the result of an inability of I-A^{g7} to associate with Ii nor H-2M. Indeed, the *H-2Ma* and *H-2Mb* genes of NOD mice exhibit very close sequence identity with those of the *d* haplotype and are, therefore, unlikely to have a direct contribution to any of the unusual features of the I-A^{g7} molecule (Hermel *et al.*, 1995). Instead, it has been proposed that the incapacity of I-A^{g7} to form significant numbers of SDS-stable dimers is yet again a consequence of the lack of an aspartic acid residue at position β57 (Nabavieh *et al.*, 1998). The structural disruption in the peptide-binding groove suggested to result from this mutation could potentially impair the ability of the I-A^{g7} heterodimer to shield its non-polar surfaces. Support for this proposal has come from the finding that the human class II MHC molecules HLA-DQ8 (βAla 57), DQ2 (βAla 57) and DQ1 (βVal 57) are also predominantly unstable in the presence of SDS detergent (Buckner *et al.*, 1996; Reizis *et al.*, 1997a). By contrast, the majority of HLA-DQ6 molecules (βAsp 57) formed SDS-resistant αβ dimers. Furthermore, stability in SDS was also shown by HLA-DQ9 (DQA1*0301/DQB1*0303; also known as HLA-DQ3.3), which differs from DQ8 by just a single amino acid residue, Ala → Asp at position β57. Nevertheless, the reintroduction into I-A^{g7} of an aspartic acid at position β57 (together with correction of the βPro56His mutation) did not enable these molecules to withstand SDS-induced dissociation (Carrasco-Marin *et al.*, 1996). Moreover, HLA-DQ7 exhibiting an aspartic acid at position β57 has also been shown to be predominantly unstable in SDS (Reizis *et al.*, 1997a) and RT1.B rat class II MHC molecules appear to be SDS-stable regardless of whether they express an aspartic acid at this position (RT1.B^{a}) or a serine (RT1.B^{l,n}; Reizis *et al.*, 1997b). Thus, although a non-Asp residue at position β57 would appear to predispose towards an inability to form SDS-stable dimers, there are clearly additional factors involved.

Lastly, in addition to upholding the global structure of the peptide-binding groove, the βAsp 57 residue may also make direct contact with the bound peptide by forming a hydrogen bond with a suitable donor group, typically a main-chain amide nitrogen. Such interactions are present in the complexes of HA–HLA-DR1 (Stern *et al.*, 1994), CLIP–HLA-DR3 (Ghosh *et al.*, 1995) and HEL–I-A^{k} (Fremont *et al.*, 1998b; Weber *et al.*, 1998). However, the exact importance of the β57 hydrogen bond for stabilising peptide binding is unclear, given that such an interaction is not apparent in the crystal structures of I-A^{d} complexed with the OVA323–339 or HA126–138 peptides (Scott *et al.*, 1998), nor in the complex between the human collagen II peptide, CII261–273, and HLA-DR4 (Dessen *et al.*, 1997). Nevertheless, it is possible that the loss of this hydrogen bonding potential in the I-A^{g7} molecule may be compensated to some extent by the presence of the polar serine residue at this position. It may be envisaged that the hydroxyl group of this residue could form an alternative hydrogen bond if an acceptor group in the peptide backbone, such as a carbonyl oxygen atom, is orientated appropriately.

6.4.5 The βPro56His mutation present in the I-A^{g7} class II MHC molecules reduces peptide exchange at the cell surface

In contrast to the effects of the βAsp57Ser mutation, the unusual presence of a histidine residue at position 56 in the I-A^{g7} β-chain did not impact directly upon the intermolecular interactions between this molecule and the CLIP ligand in this study. For example, correction of the βPro56His mutation did not alter the importance of the C-terminal CLIP side chains as anchors — the formation of favourable interactions through the residues Leu 97, Leu 98 and Arg 100–Met 102 was as important for the CLIP86–104 ligand to bind to I-A^{g7Pro56} (Figure 6.6b) as it was for the interaction with wild-type I-A^{g7} (Figure 6.6a). Moreover, both the I-A^{g7} and I-A^{g7Pro56} molecules showed the highest affinity for the longer CLIP ligands, *i.e.* those capable of providing the greatest intermolecular contact, either through a biotin moiety or additional peptide residues (Figure 6.4 & Table 6.1). This adds further support to the concept that the need for these stabilising interactions arises as a result of the loss of the β57–α76 salt bridge and is not due to the mutation of the βPro 56 residue to histidine in the wild-type I-A^{g7} molecule; a finding consistent with the orientation of the β56 residue away from the groove (*e.g.* Brown *et al.*, 1993; Stern *et al.*, 1994; Fremont *et al.*, 1998b; Scott *et al.*, 1998).

Nevertheless, although both I-A^{g7} and its single amino acid revertant form, I-A^{g7Pro56}, are similar in their need for CLIP to provide anchor interactions through its C-terminal residues, these molecules do appear to differ in their capacity for peptide exchange at the

cell surface. For example, experiments examining the direct binding of biotinylated CLIP86–104 to the cell-surface class II MHC molecules of M12.NOD, M12.PRO and M12.ASP revealed that a higher proportion of I-A^{g7Pro56} molecules were receptive for binding the biotinylated ligand than either of the other two forms, wild-type I-A^{g7} and I-A^{g7Asp57} (Figure 6.3). All three cell lines were determined to be expressing equivalent number of class II MHC molecules at the surface by OX6-FITC staining (Figure 6.2a). Furthermore, the titration of other biotinylated peptides, this time derived from protein antigens, yielded a similar result (Figures 6.7 & 6.8), *i.e.* the M12.PRO cells consistently bound more biotinylated p12(166–185), MSA560–574 and HEL10–23 peptide than the M12.NOD and M12.ASP cells, again despite all expressing similar levels of class II MHC molecules. The presence of histidine at position 56 in the β-chain of wild-type I-A^{g7} (and I-A^{g7Asp57}) thus appears to reduce the capacity of this molecule to bind peptides by exchange at the cell surface.

The flow cytometry-based assay method used in this study evaluates specifically the association of a biotinylated peptide across the total pool of cell-surface class II MHC molecules in a sample (for a discussion of this assay method, see *Chapter 3*, §3.4.1). Of these, between 1–15% have been estimated to be receptive for binding peptides, *i.e.* approximately 10^3–10^4 class II MHC molecules *per* cell (Trucco *et al.*, 1980; Watts & McConnell, 1986; Buus *et al.*, 1988; Busch *et al.*, 1990; Roche & Cresswell, 1990b; Ceppellini *et al.*, 1989). One feature to influence significantly the level of fluorescence signal obtained in such experiments is the composition of peptide ligands already bound. Given that I-A^{g7} appears to bind less exogenously-added peptide than its single amino acid revertant form, I-A^{g7Pro56}, it may therefore be envisaged that more of the wild-type heterodimers may have ligand already bound tightly within the groove. Alternatively, I-A^{g7} may be generally unreceptive to binding peptides at the cell surface as a consequence of an inherent structural defect in the αβ complex.

Conflicting evidence exists concerning the possibility that the majority of I-A^{g7} class II MHC molecules may be loaded with peptides in stable complexes at the cell surface. On the one hand, Reich *et al.* (1994) have eluted successfully a range of different naturally-processed peptides from these molecules and shown subsequently their capacity to rebind as synthetic analogues. On the other hand, however, Reizis *et al.* (1997b) have reported that the overall yield of peptides isolated from purified I-A^{g7} is very low compared with that from other class II MHC molecules. Indeed, these latter authors have put forward the suggestion that a large fraction of these molecules at the cell surface may in fact be empty; a scenario which would be expected to lead to higher levels of peptide binding at the cell surface. In direct conflict to the findings of the study presented herein, this claim was

supported by the report that the p12 peptide bound to I-A^{g7} at the surface of fixed APC at lower concentrations than required for other peptide–I-A combinations (Reizis *et al.*, 1997b). In this respect, it is possible that the findings of Reizis *et al.* (1997b) may be misleading as a result of these authors making comparisons between individual I-A allotypes binding different peptides. This problem has been avoided in this study by taking advantage of the promiscuous nature of the CLIP86–104 ligand to examine the interaction of this single peptide with the different class II MHC variants. Furthermore, the interpretations presented herein regarding the extent of cell-surface peptide exchange have been restricted to closely-related class II MHC molecules, specifically, I-A^{g7} and its two revertant forms, I-A^{g7Pro56} and I-A^{g7Asp57}, which differ from the wild-type heterodimer by just a single amino acid at position β56 or β57.

Although seeming to conflict with the findings reported in this study, the suggestion by Reizis *et al.* (1997b) that many I-A^{g7} dimers may be devoid of peptide ligand at the cell surface is not without merit, since it would appear to be more in keeping with the very restricted range of peptides expected to have the capacity to form optimal interactions with these molecules, as described in §6.4.4. Indeed, it might be imagined that many of the peptides that associate initially with this particular class II MHC molecule are ultimately unable to form sufficient bonds to stabilise the association and thus eventually dissociate to leave the peptide-binding groove empty; a potential explanation for the reduced lifespan of some peptide–I-A^{g7} complexes reported by Carrasco-Marin *et al.* (1996). In support of this, the majority of peptides eluted from I-A^{g7} were shown to exhibit the preferred acidic residue with the C-terminus, the major contributor to forming stable associations with this molecule (Reich *et al.*, 1994).

It is interesting to note that CLIP was not amongst the peptides eluted from I-A^{g7}, a finding reminiscent of peptide elution studies of I-E class II MHC molecules (*Chapter 5*, §5.4.4). Unlike the situation with I-E, however, it has been shown that CLIP binds to I-A^{g7} better at neutral pH than in acidic conditions, ostensibly as a consequence of a less pronounced preference for an acidic residue at P9 in this environment (Reizis *et al.*, 1997b). Similarly, Hausmann *et al.* (1999) have reported that CLIP–I-A^{g7} complexes that are formed at neutral pH dissociate rapidly at pH 5.0, but this particular result should be considered with some caution since the CLIP ligand used to show this, residues 87–101, lacked the Met 102 residue identified in this study to provide critical anchoring contacts with the αβ dimer (Figure 6.6) and it remains unknown whether this truncated ligand is produced *in vivo*. Indeed, the same peptide, CLIP87–101, did not bind to the wild-type I-A^{g7} molecules at neutral pH in the cell-surface peptide binding assays presented herein (Table 6.3). Nevertheless, CLIP has been reported to bind optimally to HLA-DQ8

molecules (βAla 57) also at a less acidic pH than other peptides (Buckner *et al.*, 1996). Thus, the lower overall degree of competitive CLIP86–104 binding to the wild-type I-A^{g7} molecules in this study (Figure 6.4) does not appear to arise as a result of the interactions being unfavourable in the neutral conditions under which these experiments were conducted. Consistent with this, the same effect was observed for the association of wild-type I-A^{g7} with the antigenic peptides, p12(166–185), MSA560–574 and HEL10–23, the former two exhibiting the preferred acidic P9 residue (Figures 6.7 & 6.8d).

Instead, the proposed inability of many peptides to form stable interactions with I-A^{g7} and, related to this, the reduced lifespan of some peptide–I-A^{g7} complexes (both discussed in §6.4.4), both suggest a potential explanation for the reduced cell-surface peptide binding observed in this study for I-A^{g7}. Class II MHC molecules devoid of peptide ligand have been reported to undergo a rapid conformational rearrangement into a form in which they are no longer "active" for binding peptide. To become receptive again, the αβ dimer must undergo a very slow conformational reversion. This phenomenon has been so far been observed in the molecules, I-E^{k} (Rabinowitz *et al.*, 1998) and HLA-DR (Natarajan *et al.*, 1999a), and some evidence also exists to suggest that it may take place in I-A^{k} and I-A^{u} (Mason *et al.*, 1995). If ligands tend to dissociate from I-A^{g7} more readily than from other class II MHC molecules, then it is possible that a high number of these expressed at the cell surface are also in an inactive form. This would indeed result in the total pool of I-A^{g7} molecules at the plasma membrane binding exogenously-added peptides to a lesser extent than other allotypes, as observed in this study (Figures 6.3, 6.7 & 6.8). Moreover, this theory reconciles the finding of a low yield of peptides eluted from these molecules (Reizis *et al.*, 1997b) with the reduced amount of cell-surface binding of antigen-derived peptides, even those that exhibit an acidic residue at P9 (Figure 6.7 & 6.8).

The inability to elute CLIP ligands from I-A^{g7} αβ dimers raises the possibility that even this peptide may ultimately lack the ability to maintain a long-term association with these molecules at the cell surface, despite the additional stabilising interactions that take place through its C-terminal side chains. This would also have a significant impact on the extent of cell-surface peptide exchange *via* these molecules. Specifically, CLIP–class II MHC complexes arise as natural biosynthetic intermediates following the proteolytic degradation of the invariant chain protein and a small percentage of these appear to traffic to the cell surface (discussed previously in *Chapter 5*, §5.4.5). Whether this occurs by error or as a mechanism to broaden the spectrum of antigenic peptides to be presented to T cells, the cell-surface CLIP–αβ complexes typically represent a major source of the molecules susceptible to peptide exchange (Riberdy *et al.*, 1992; Griffin *et al.*, 1997). If,

however, this ligand ultimately dissociates from I-A^{g7} before more thermodynamically-favoured peptides are available, as implied by the inability to elute CLIP from these molecules, then the population of I-A^{g7} heterodimers at the cell surface to which exogenously-added peptide normally binds may be reduced considerably. An analysis of the relative amounts of CLIP present at the cell surface in association with I-A^{g7} compared with other class II MHC I-A allotypes is currently under way in this laboratory, as part of the doctoral research of Ms A. Bhatnagar.

One interesting finding of the study presented herein is that the reduced capacity of I-A^{g7} molecules to bind exogenously-added peptides appears to be a consequence, not of the βAsp57Ser mutation in these molecules, but of the βPro56His mutation. In this respect, it may be envisaged that, while the β57 mutation in I-A^{g7} is responsible for the selectivity of this molecule in terms of what peptides may bind, it is ultimately the β56 mutation that makes the molecules unreceptive to binding peptides at the cell surface. In this sense, if a histidine residue at position β56 somehow reduces cell-surface peptide binding then, by extension, the proline residue normally present in class II MHC αβ dimers at this position must play a role in keeping these molecules receptive at the plasma membrane. As mentioned previously (§6.1.5), this residue lies within the H1 α-helix of the β1 domain which contributes to the walls of the binding cleft (Brown *et al.*, 1993; Stern *et al.*, 1994; Fremont *et al.*, 1998b; Scott *et al.*, 1998). The restricted rotational angles of the proline amino acid are likely to confer a peculiar spatial arrangement of atoms at this site within the helix which appears to maintain this region of the peptide-binding groove both suitable for peptide binding and peptide exchange. It is probable that the replacement of the βPro 56 residue by a histidine in the I-A^{g7} dimer would cause a very different ordering of the atoms in the three-dimensional space around this site and affect the coiling of the H1 α-helix. Indeed, a conservative amino acid substitution at another residue within this same helix, position β61, has been shown to change the configuration of this region of the peptide-binding groove such that the formation of at least one hydrogen bond with the peptide ligand is affected and T cell recognition of the complex is impaired (Tate *et al.*, 1995). The resulting change in the conformation of this region of the I-A^{g7} peptide-binding groove due to the βPro56His mutation may make this molecule more susceptible to peptide dissociation or to undergoing rearrangement into the inactive form once the peptide has dissociated. This is contrary to a proposal by Reizis *et al.* (1997b) that I-A^{g7} molecules may be unusually stable in the absence of bound peptide through the formation of a unique interaction between residues αGlu 66 and βHis 9. The consequences of these possibilities upon antigen presentation in the NOD mouse are discussed subsequently in §6.4.6.

The findings of this study regarding the extent of peptide binding to cell-surface class II MHC molecules rely upon the assumption that the OX6 monoclonal antibody used to measure the relative levels of these molecules recognises each of the three I-A^{g7} forms with a similar efficiency. This is indeed likely to be the case, since this mAb is known to recognise a sequential epitope of the β-chain that is common to many class II MHC molecules, *e.g.* I-A^{k,u,r,s,f} (McMaster, 1981; Reske & Weitzel, 1985; Liu *et al.*, 1993), some of which also exhibit the particular polymorphisms observed in I-A^{g7} (Liu *et al.*, 1993). Moreover, OX6 binds to the RT1.B^{l} molecules of Lewis rats which exhibit a serine at position β57 (Reske & Weitzel, 1985) but also recognises I-A^{k} which exhibits the more typical aspartic acid at this site (Liu *et al.*, 1993). In this respect, it is interesting to note that many of the mAb that react with I-A^{g7} also cross-react with I-A^{k,u,r,s,f} molecules (*e.g* 10-2.16, 10-3.6.2; Sher *et al.*, 1984) but, surprisingly, I-A^{g7} is rarely recognised by mAb raised to I-A^{d}, *e.g.* MK-D6 (Hattori *et al.*, 1986; Liu *et al.*, 1993), despite these two molecules exhibiting greater sequence homology (Acha-Orbea & McDevitt, 1987). The molecular basis of this property appears to be a two-residue insertion relative to I-A^{g7} at positions β65 and β66 in the I-A^{d} heterodimer (Liu *et al.*, 1993; numbered as according to the secondary structure-based sequence alignment of the α_1 and β_1 domains of the molecules, I-A^{k}, I-E^{k} and HLA-DR, reported by Fremont *et al.*, 1998b). In the X-ray crystallographic structure of I-A^{d}, these two residues fall at the junction of the H1 and H2a helices of the β-chain, their side chains extending upwards from the plane of the binding cleft to represent potential TCR contact sites (refer to *Chapter 1*, Figure 1.4; Scott *et al.*, 1998). The projection of these residues from the main structure of the peptide-binding groove also makes them potential determinants for recognition by mAb. Indeed, the sequence spanning the residues, β63–67, has been identified as critical to the binding of the I-A^{d}-reactive mAb, MK-D6 (Braunstein *et al.*, 1990), hence providing an explanation for the inability of this mAb to cross-react with I-A^{g7}.

The relative insertions in the I-A^{d} protein at positions β65 and β66 are also present in I-A^{p} and I-A^{q} and, accordingly, these molecules are also recognised by MK-D6 (Liu *et al.*, 1993). An examination of the aligned amino-acid sequences of the β-chains of I-E and HLA-DR variants reveals that equivalent residues are present in these molecules too (Fremont *et al.*, 1998b). By contrast, I-A^{g7} is more similar to I-A^{k,u,r,s,f}, all of which lack these residues (Liu *et al.*, 1993). In correspondence with this, none of these molecules are detected by MK-D6 (Liu *et al.*, 1993) although all are recognised by the mAb, 10-3.6.2 and 10-2.16 (Sher *et al.*, 1984). The specific residues bound by these latter two mAb still remain to be mapped but it is known that they exist in a single linear sequence upon the β-chain (Sher *et al.*, 1984). In this respect, it is tempting to speculate that their shared epitope resides in the same stretch of polypeptide as that of the MK-D6 mAb,

thereby providing an explanation for the ability of these monoclonal antibodies to distinguish class II MHC molecules based upon the whether they exhibit the relative insertions at positions, β65 and β66. Indeed, these insertions do appear to cause a significant structural difference: when compared with the crystal structures of I-E^{k} (Fremont *et al.*, 1996), HLA-DR1 (Stern *et al.*, 1994), HLA-DR4 (Dessen *et al.*, 1997) HLA-DR3 (Ghosh *et al.*, 1995) and I-A^{d} (Scott *et al.*, 1998), the crystal structure of I-A^{k} shows a region of significant structural divergence around the H1 helix of the β-chain and its junction with the adjoining H2a helix (Fremont *et al.*, 1998b). Fremont *et al.* (1998b) have calculated that the change in this region in I-A^{k} translates to a 1.5 Å shift in the C-terminal half of the H1 helix towards the H2a helix relative to the equivalent residues in I-E^{k} or HLA-DR. Related to this, it is interesting to note that the only difference perceived between the crystal structures of the SDS-unstable CLIP–DR3 complex and the SDS-stable HA–DR1 complex was a 1.5 Å shift towards the peptide-binding groove of a small segment of the β-chain α-helix between the residues, β65–β74 (Ghosh *et al.*, 1995). If the different three-dimensional arrangement of these residues has any relevance upon the behaviour of these complexes in the presence of SDS detergent, one must wonder how the relative deletions of the β65 and β66 residues in the molecules I-A$^{g7.k.q.r.s.f}$ impact upon this.

Lastly, there exists a possibility that the βPro56His mutation present within I-A^{g7} may contribute a further preference for C-terminal acidic residues in peptide ligands through the introduction of a positively-charged side chain at this site (discussed previously in §6.1.5). In this study, the rMOG8–22 peptide, which exhibits a cluster of acidic residues at the positions, P9, P10, P12 and P13 in its C-terminus (Figure 6.8a), bound less to the I-A^{g7Pro56} mutant than to the wild-type I-A^{g7} molecule (Figure 6.8c). One could speculate that this ligand forms a stabilising interaction with the I-A^{g7} αβ dimer between one of its other C-terminal acidic residues and the βHis 56 residue. The reversion to the proline residue at β56 in I-A^{g7}, *i.e.* to form I-A^{g7Pro56}, thus removes this favourable charge interaction thereby negatively affecting the ability of this peptide to compete for binding.

6.4.6 The unusual properties of I-A^{g7} may alter CD4^{+} T cell activities both during thymic selection and within the periphery

The amino acids unique to the I-A^{g7} heterodimer at positions β56 and β57 have been shown previously to influence the diabetogenic potential of this class II MHC molecule in the NOD mouse (Lund *et al.*, 1990a; Quartey-Papafio *et al.*, 1995; Singer *et al.*, 1998). Accordingly, the findings of this study have a number of implications for the functioning

of CD4$^+$ T lymphocytes *in vivo*, the cellular population ultimately responsible for the development of autoimmune diabetes in these animals.

Of major significance, the results of this investigation support the possibility that the I-A^{g7} heterodimer may direct the intrathymic selection of a unique CD4$^+$ T cell repertoire in NOD mice. Indeed, there exist a number of possibilities by which the biochemical peculiarities of the I-A^{g7} molecule may modulate the outcome of any given MHC–TCR interaction within the thymus when viewed in the context of the differential avidity model of T cell selection. As described in *Chapter 1* (§1.7.2), this model proposes that the fate of an immature thymocyte is decided by the product of the affinity of the TCR for the peptide–MHC complex, the affinity of the peptide for the MHC molecule, the concentration of the peptide–MHC complex on the APC and the number of TCR on the thymocyte (Ashton-Rickardt *et al.*, 1994; Ashton-Rickardt & Tonegawa, 1994). Specifically, T cells undergo positive selection when the overall avidity of the interaction of their TCR with a peptide–MHC complex is above a certain threshold, but if this avidity is too high, the thymocyte is targeted for deletion during negative selection.

Firstly, peptides exhibiting acidic P9 anchors (as discussed in §6.4.3 & §6.4.4) will be likely to predominate in the binding cleft of I-A^{g7} molecules at the cell surface. As a result, such complexes will represent the most probable targets for high avidity TCR interactions thereby skewing negative selection towards the deletion of thymocytes recognising primarily these peptides. At the same time, the generally poor ability of a broad range of other peptides to form highly stable complexes with I-A^{g7} (discussed in §6.4.4) will mean that many potentially self-reactive T cells that are normally deleted in other mouse strains may escape into peripheral circulation in the NOD mouse. An equivalent effect has been observed in H-2M knockout mice where the occupation of most class II MHC molecules by CLIP fragments prevents presentation of other self-antigens necessary for the induction of self-tolerance. Accordingly, CD4$^+$ T lymphocytes derived from H-2M^o animals react strongly with syngeneic APC presenting a wild-type peptide repertoire (Surh *et al.*, 1997; Tourne *et al.*, 1997; Lee *et al.*, 1999). A similar result was obtained with T cells from the so-called A^{b}Eαp mice which have been engineered to express just a single peptide–class II MHC complex of residues 52–86 of the I-Eα polypeptide covalently linked to the β-chain of I-A^b (Ignatowicz *et al.*, 1996).

It is possible that NOD mice may exhibit an even more significant defect in the positive selection of T cell specificities. Indeed, this is supported by the demonstration that the NOD thymic epithelium alone is sufficient to select an autoimmune T cell repertoire when grafted into athymic mice of the non-autoimmune-prone strain, C57BL/6 (Thomas-Vaslin

et al., 1997). In this respect, the limited peptide-binding specificities of the I-A^{g7} heterodimer, as demonstrated in this study (§6.4.3 & §6.4.4), may also have significant consequences upon the positive selection of immature thymocytes. To date, a number of investigators have shown a strong correlation between the extent of peptide diversity in the thymus and the breadth of the TCR repertoire (Ashton-Rickardt *et al.*, 1994; Hogquist *et al.*, 1994; Bevan, 1997; Grubin *et al.*, 1997; Surh *et al.*, 1997; Barton & Rudensky, 1999; Janeway, 1999). Regarding NOD mice, the inability of many peptide ligands to fulfil the motif requirements of I-A^{g7} may mean fewer thymocytes will fulfil the avidity threshold to undergo positive selection. As a result, the collection of CD4$^+$ T lymphocytes present in these animals may exhibit a number of "holes", possibly including TCR specificities involved in essential immunoregulatory functions. This feature may be exacerbated further by the impaired ability of I-A^{g7} molecules at the cell surface to undergo ligand exchange (discussed in §6.4.5). Again, an equivalent effect has been observed in H-2M° and A^{b}Eαp mice whereby certain TCR specificities that are present in normal mouse strains are not selected to mature in these animals (Ignatowicz *et al.*, 1996; Grubin *et al.*, 1997; Surh *et al.*, 1997; Tourne *et al.*, 1997).

In concert with the peptide-specificity effects, particular molecular aspects of the I-A^{g7} heterodimer may also influence which thymocytes will mature into immunocompetent CD4$^+$ T cells in the NOD thymus. For example, the loss of the β57–α76 salt bridge is predicted to cause localised destabilisation within the heterodimer (Nalefski *et al.*, 1995) and may lead to peptide–I-A^{g7} complexes which have unusually short half-lives (Carrasco-Marin *et al.*, 1996, 1997; discussed previously in §6.4.4). Together, these events may be envisaged to reduce the capacity of a TCR to engage such a complex for sufficient duration to allow the necessary signals to be transduced within the thymocyte to direct an appropriate outcome.

Another possible disturbance in the selection of the CD4$^+$ T cell repertoire in NOD mice may arise from the conformational abnormalities predicted in I-A^{g7} due to the unique sequence at the β56 and β57 positions. Given the proximity of these positions to the peptide-binding groove, any alterations in the spatial arrangement of the surrounding atoms may have effects on both the ability of a given peptide to bind to this heterodimer and on the recognition of this complex by different TCR. In this respect, Chervonsky *et al.* (1998) have shown recently that different T cells may discriminate even between very subtle conformational changes introduced into a single class II MHC molecule by the binding of different peptides. Moreover, Yassine-Diab *et al.* (1999) have reported that polymorphism in the peptide-binding groove may bias the selection of particular TCR variable domain gene segments even when peptide binding is unaffected by these amino

acid changes. Regarding NOD mice, T cell clones have been identified that recognise peptides complexed with I-A^{g7} but not the same peptides bound to either its double revertant form, I-A^{g7Pro56Asp57}, nor to I-A^{d} (Kanagawa *et al.*, 1997). Similarly, T cell clones have been isolated from NOD.ASP mice restricted to I-A^{g7Asp57} but which show no cross-reaction with wild-type I-A^{g7} (Quartey-Papafio *et al.*, 1995). CD4^{+} T cell clones have also been reported with the ability to differentially recognise HLA-DQ allotypes based solely upon the identity of the residue present at position β57 (Lundin *et al.*, 1988; Sterkers *et al.*, 1988; Kwok *et al.*, 1996b). Molecular modelling of a number of HLA-DQ variants has revealed interesting differences in the surface contours of the binding clefts of these molecules which appear to be aligned with their relative genetic associations with the development of IDDM (Hoover & Marta, 1997; Sanjeevi *et al.*, 1997). These common features were not limited solely to the effects of polymorphism at position β57 but may induce the selection of a novel repertoire of CD4^{+} T cells in IDDM-susceptible individuals, possibly enriched in autoaggressive specificities or, alternatively, lacking those associated with the control of the thymocyte population in the periphery.

The efficiency of T cell selection is likely to be impaired further if the cell-surface expression of I-A^{g7} is reduced, as suggested by the results of site-directed mutagenesis of I-A^{d} in which the formation of the β57–α76 salt bridge was disrupted (§6.4.4; Nalefski *et al.*, 1995). As mentioned above, the number of peptide–I-A^{g7} complexes on the cell surface is a critical parameter in determining the fate of T cells during positive selection (Ashton-Rickardt *et al.*, 1994; Ashton-Rickardt & Tonegawa, 1994). The outcome on T cell development of defective cell-surface expression of class II MHC molecules has been illustrated previously in mice that exhibit a deletion of the Ii gene. The lack of the Ii protein to co-ordinate the intracellular trafficking of the αβ dimers means that such molecules only rarely reach the plasma membrane (Bikoff *et al.*, 1993; Viville *et al.*, 1993; Elliott *et al.*, 1994a; described in *Chapter 1*, §1.4.2). Moreover, of those that do achieve cell-surface expression in Ii^{0} mice, the peptide repertoire that they present appears to be altered significantly and the complexes may exhibit atypical conformational states. Similar to the effects described above for the H-2M knockout and A^{b}Eαp mice, CD4^{+} T lymphocytes derived from animals lacking Ii have been shown to be diminished in numbers and lack a number of the TCR specificities found in normal mice (Bikoff *et al.*, 1993; Viville *et al.*, 1993; Tourne *et al.*, 1995).

All of the potential effects of the I-A^{g7} heterodimer upon the intrathymic selection of TCR specificities within the NOD mouse are related outcomes from the presence of the amino acids, βHis 56 and βSer 57, of this atypical class II MHC molecule. There exists also the strong possibility that the unusual biochemical features of peptide–I-A^{g7} complexes may

render these molecules incapable of interacting effectively with immature thymocytes such that even those T cells with the highest avidity reactivities for self-antigen are not deleted efficiently in these animals, *i.e.* those CD4$^+$ T cells that exhibit specificities for peptides exhibiting acidic P9 anchors. This proposal is supported by reports of a high incidence of autoreactive T cells in NOD mice (Ridgway *et al.*, 1996; Carrasco-Marin *et al.*, 1997). However, T cells that recognise self-peptides have also been described in other mouse strains (Schild *et al.*, 1990; Agrawal *et al.*, 1991; Oldstone *et al.*, 1991; Guerder *et al.*, 1994). What appears to be unusual in NOD mice is that a proliferative CD4$^+$ T cell response is able to be stimulated readily by immunising with a number of different self-antigens (Ridgway *et al.*, 1996), an effect not generally observed in other mouse strains (Ridgway *et al.*, 1996; Ridgway & Fathman, 1998). This may be indicative of an immune regulatory defect in NOD mice, a possible consequence of lacking a certain regulatory T cell subset, as discussed previously (§6.1.3).

Another interesting possibility is that the self-reactive T cells that escape into peripheral circulation in NOD mice may exhibit higher affinities for their self-antigen–MHC targets than those present in other mouse strains. For example, it may be envisaged that to achieve the avidity threshold for positive selection in NOD mice, T cells are selected that exhibit higher than usual affinities for self-antigens in order to compensate for the generally diminished ability of I-A^{g7} molecules to form stable peptide complexes. This prospect has been reviewed by Ridgway & Fathman (1998). Similarly, only the very highest affinity T cells would exceed the avidity threshold above which negative selection is triggered. In this respect, it is interesting to note that the dominant T cell epitope of rat myelin basic protein (MBP), r*Ac*1–11, which induces EAE in susceptible mice, exhibits a very low affinity for its restriction element, I-A^u, thereby enabling encephalitogenic T cells to escape tolerance induction in the thymus (Fairchild *et al.*, 1993). However, the intraperitoneal administration of substituted analogues of this peptide that form more stable complexes with the I-A^u molecule induces the deletion of these specificities and the animals become less susceptible to disease (Liu *et al.*, 1995; Liu & Wraith, 1995).

Autoimmune disease in an individual is not triggered simply by the release of autoreactive T cells from the thymus into circulation. The destruction of host tissues occurs only after these cells become activated by recognising antigen presented by MHC molecules in the periphery. Like the selection of TCR specificities, the efficiency with which mature T cells are activated has also been proposed to operate by a differential avidity mechanism — interrelationships appear to exist between the numbers of TCR on the T cell, the peptide–MHC concentration on the APC, the affinity of the TCR for this complex and the affinity of the MHC molecule for the peptide (*Chapter 1* , §1.7.1; Kim *et al.*, 1996; Alam

et al., 1999; Legge *et al.*, 1999). An effector response is induced when the overall avidity of the interaction between a T cell and a particular peptide–MHC combination is sufficiently high to trigger and subsequently downregulate a certain number of TCR (Viola & Lanzavecchia, 1996; Lanzavecchia, 1997; Lanzavecchia *et al.*, 1999).

Regarding NOD mice, it is likely that the same features of the atypical I-A^{g7} class II MHC molecule that influence the repertoire development of the CD4$^+$ T lymphocyte population in the thymus will also impact significantly upon the functioning of these cells in circulation. For example, as in the thymus, I-A^{g7} molecules in the periphery would be expected to present a unique array of peptides by consequence of their unusual preference for a negatively-charged P9 anchor (§6.4.3). This collection of ligands may include potential immunopathogenic determinants, unable to bind significantly to class II MHC allotypes of other mouse strains as a result of the contrasting binding motifs. In this respect, this suggestion is consistent with the determinant selection model of autoimmunity (Smilek *et al.*, 1990). Indeed, I-A^{g7} is the only one of ten different mouse class II MHC allotypes tested that exhibits the ability to present the GAD65 determinant, residues 255–269, and the closely-related peptide, residues 32–47, of the Coxsackie B4 viral protein, P2-C, both of which exhibit an acidic residue within their C-terminal region and have been implicated in the development of this disease (Tian *et al.*, 1994; described previously in §6.1.3). However, the propensity for autoimmunity to develop in NOD mice is likely to be increased further by the effects of the other biochemical peculiarities of the I-A^{g7} heterodimer upon the functioning of the T cell repertoire, *i.e.* the likely difference in the conformational arrangement of atoms around the β56 and β57 residues, the potential reduction in numbers of peptide–I-A^{g7} complexes expressed on APC (Nalefski *et al.*, 1995) and the possibility that such complexes may exhibit short half-lives (Carrasco-Marin *et al.*, 1996, 1997). Collectively, these properties of the I-A^{g7} αβ dimer may compromise the capacity of this molecule to elicit the appropriate response when interacting with peripheral T cells.

6.5. Conclusions

In this study, the possible molecular mechanisms that underlie the genetic association of the class II MHC molecule, I-A^{g7}, with autoimmune disease have been investigated. This has been achieved by assessing the ability of different substituted and/or length-altered CLIP analogues to compete against biotinylated CLIP86–104 in cell-surface binding assays using the I-A^{g7}-expressing B lymphoma cell line, M12.NOD. The specific contributions of the two unusual I-A^{g7} polymorphisms, βPro56His and βAsp57Ser, on

peptide interactions have also been dissected by examining the binding of the CLIP analogues to two further cell lines, M12.PRO and M12.ASP, each expressing a single amino-acid revertant form of the I-A^{g7} class II MHC molecule, I-A^{g7Pro56} and I-A^{g7Asp57}, respectively (Quartey-Papafio *et al.*, 1995).

From the results of these experiments, it is apparent that the binding of CLIP to I-A^{g7} is supported by a network of hydrogen bonds between the peptide backbone and the residues of the class II MHC groove, in a fashion similar to that found in the association of this ligand with I-A^{a}, I-A^{k} and I-A^{d} (*Chapter 4*). However, the interaction of CLIP with I-A^{g7} exhibits some important differences. For example, binding to the I-A^{g7} αβ dimer is dependent upon a number of critical anchor contacts made through the C-terminal residues of the CLIP sequence, Leu 97, Arg 100 and Met 102. Additional positive binding energy is provided through the side chains of Leu 98 and Pro 101. However, of these favourable side-chain interactions, only that *via* Leu 97 is predicted to take place by conventional means within a pocket of the I-A^{g7} peptide-binding groove.

The requirement for additional positive interactions to uphold the binding of CLIP to I-A^{g7} appears to be a consequence of the βAsp57Ser mutation in the heterodimer, since the extent to which these contacts form may be modulated by the reintroduction of such a residue. This is consistent with the participation of βAsp 57 in other class II MHC αβ dimers in a salt bridge with αArg 76 that stabilises the region of the binding groove in the vicinity of the peptide C-terminus. The absence of an aspartic acid residue at position β57 also confers upon the I-A^{g7} molecules a preference to bind peptides that exhibit a negatively-charged residue at the P9 position, in order to provide a compensatory electrostatic interaction with the unpaired αArg 76 side chain, *e.g.* the CLIP M99E analogue. However, such peptides are unable to bind to the I-A^{g7} molecule when βAsp 57 is reintroduced, *i.e.* I-A^{g7Asp57}, and similarly, to other mouse I-A class II MHC molecules that exhibit the conserved βAsp 57 residue. Meanwhile, the lack of a proline at position 56 in the β-chain of I-A^{g7} leads to inefficient peptide exchange at the cell surface, such that exogenously-supplied peptides do not bind to the same extent to the wild-type heterodimer as to I-A^{g7Pro56}.

Thus, the mutations at the β-chain residues 56 and 57 in the I-A^{g7} molecule of NOD mice both influence the binding of CLIP to this αβ dimer, compared with other class II MHC molecules of the I-A isotype. These findings provide reasonable evidence to suggest that, in NOD mice, the manner in which peptide antigen is presented by the I-A^{g7} class II MHC molecules to the TCR of CD4^{+} T lymphocytes is also unique. For example, only a restricted range of peptides are likely to exhibit the structural features suitable to be

accommodated stably within the peptide-binding groove of this molecule. These properties of I-A^{g7} would be expected to have significant consequences upon CD4$^+$ T lymphocyte activities, both in the thymus during the development of the T cell repertoire and, later, within the periphery. In this way, these mutations may well contribute to development of autoimmunity in these animals.

CHAPTER 7.

Final discussion

The major focus of this study was to elucidate the molecular mechanisms by which the phylogenetically-conserved, Ii-derived peptide, CLIP, is able to bind promiscuously to different mouse class II MHC molecules. Early in this investigation, the X-ray crystallographic analysis of a human CLIP ligand complexed with HLA-DR3 confirmed that this interaction takes place within the MHC peptide-binding groove, the same site at which fragments of protein antigens bind for cell-surface presentation to $CD4^+$ T lymphocytes (Ghosh *et al.*, 1995). The results of this study have shown that CLIP from mouse Ii is able to associate with different I-A and I-E class II MHC molecules, irrespective of their individual peptide-binding motifs, by consequence of a unique mode of interaction. Firstly, the predominant source of positive binding energy is an intermolecular hydrogen bond network, presumably similar to that observed in the CLIP–HLA-DR3 crystal structure (*Chapter 4*, §4.4.1 & *Chapter 5*, §5.4.1; Ghosh *et al.*, 1995). This is able to form independently of the peptide amino acid sequence because it involves only the main-chain atoms. Secondly, allele-specific anchor contacts within the polymorphic pockets are avoided in favour of a small number of atypical favourable interactions between CLIP side chains and regions of the groove between the classical pockets that are conserved among allotypes (*Chapter 3*, §3.4.4 & *Chapter 5*, §5.4.2). This is a novel finding. Additionally, contacts have been identified between CLIP and the αβ dimer outside the peptide-binding groove. These occur at both the N- and C-termini. In particular, the previously undescribed positive interaction deriving from the side chain of Met 102 plays an important role in stabilising the binding of mouse CLIP to class II MHC molecules. The side chain at this position in human CLIP is leucine and it remains to be determined whether Leu 102 is important in this system as well.

This study has also identified a number of inhibitory contacts that may be present in the binding of CLIP to mouse class II MHC molecules. These are typically a consequence of steric conflict between the residues of the peptide-binding groove and the bulky side chains of the proline residue at position 96 and/or one of the three methionines within this peptide sequence (*Chapter 3*, §3.4.4 & *Chapter 5*, §5.4.2). Incorporating these unfavourable interactions into the general binding motif of CLIP proposed above, it may be envisaged that this ligand exhibits an intermediate affinity for the different αβ dimers. This is of fundamental importance in the pathway of class II MHC antigen presentation. For example, it is critical that this affinity is not so high as to hinder the replacement of CLIP by suitable antigen-derived peptides with appropriate anchors in the presence of HLA-DM/H-2M (Gautam *et al.*, 1997). However, the CLIP region represents the primary site of interaction between intact Ii and class II MHC molecules (Freisewinkel *et al.*, 1993; Biljmakers *et al.*, 1994a). Therefore, the affinity of this sequence also needs to be of sufficient strength to uphold this interaction to enable Ii to target the αβ dimers from

the ER into the endocytic route and, similarly, for the isolated CLIP fragments to maintain the binding groove in a receptive state following the proteolytic degradation of Ii (Mason *et al.*, 1995; Rabinowitz *et al.*, 1998; Natarajan *et al.*, 1999a).

Some isotypic differences in the manner in which CLIP binds to I-A and I-E molecules have been observed in this study. For example, CLIP was found to be unable to sustain an association with I-E molecules at neutral pH although binding to I-A molecules was detected under the same conditions (*e.g. Chapter 5*, Figure 5.3). I-E molecules typically display a lower optimum pH for binding peptides than those of the I-A isotype as a result of high proton concentration facilitating the formation of a number of hydrogen bonds in these complexes additional to those in common with peptide–I-A complexes (Jensen, 1990; Mouritsen *et al.*, 1992; Reay *et al.*, 1992; Sette *et al.*, 1992b; Fremont *et al.*, 1996; Wilson, 1996). This property may have functional significance in the nature of the antigen-derived ligands typically bound by the I-A and I-E mouse class II MHC heterodimers by influencing the site within the endocytic route at which these molecules form stable complexes with cognate peptides (Jensen, 1991; Sette *et al.*, 1992b). Also of note, the few favourable contacts made by CLIP side chains with these molecules are considerably stronger for I-E^d $\alpha\beta$ dimers so as to represent anchor interactions (*Chapter 5*, Figure 5.8). By comparison, an equivalent effect was observed only for one residue in the association of CLIP with the I-A^d allotype under the same experimental conditions at pH 5.0 (*Chapter 3*, Figure 3.6). The presence of several anchor contacts may be a general feature of the interaction of CLIP with molecules of the I-E isotype. The evolution of this binding mechanism may have been facilitated by the non-polymorphic I-Eα polypeptide (Ayane *et al.*, 1986).

The role of the N-terminal residues of CLIP in modulating the affinity of this peptide for different mouse class II MHC molecules requires further investigation. For example, the binding of CLIP to I-A^s was enhanced at pH 7.0 when either of the residues, Lys 83 and Pro 87, were substituted with L-alanine (*Chapter 4*, Figures 4.10 & 4.11). By comparison, these same modifications to the CLIP sequence resulted in a decrease in the binding to I-E^d molecules at pH 5.0 (*Chapter 5*, Figure 5.9). It may be misleading to attempt to interpret these findings in terms of either the ability of the Ii-4 peptide (CLIP77–92) to promote peptide exchange from I-E molecules at the cell surface (Adams & Humphreys, 1995) or the self-release of human CLIP from HLA-DR molecules (Kropshofer *et al.*, 1995b), since different experimental approaches were used in each case. In particular, there exist inconsistencies in the pH at which the experiments were performed and the data of Kropshofer *et al.* (1995b) have assessed specifically the kinetic

properties of this interaction whereas the results presented herein reflect thermodynamic stability.

The capacity of particular N-terminal residues within the CLIP sequence to modulate the binding affinity of this ligand for different class II MHC molecules is certainly interesting given that these residues are not all accommodated within the peptide-binding groove. Indeed, only 15 of the 24 residues of the human CLIP81–104 peptide that were crystallised in association with the HLA-DR3 molecule showed clear, interpretable electron density indicative of binding in an ordered manner within this site, namely residues Pro 87–Ala 101 (Ghosh *et al.*, 1995). The remaining outermost amino- and carboxyl-terminal residues protrude from the groove in a manner facilitated by its being open at both ends. In this sense, the ability of intermediate-length CLIP ligands to bind better to some class II MHC molecules than longer variants (Chicz *et al.*, 1992; Urban *et al.*, 1994; Geluk *et al.*, 1995; Naujokas *et al.*, 1998) makes it interesting to speculate that, should such peptides occur *in vivo* through the actions of aminopeptidases, then the efficient displacement of CLIP in the endosomes may be hindered and antigen presentation impaired. Fortunately, however, an examination of the naturally-processed Ii peptides eluted from different αβ dimers reveals that such N-terminally-truncated CLIP ligands are not generated frequently *in vivo* (Rudensky *et al.*, 1991; Chicz *et al.*, 1992, 1993; Hunt *et al.*, 1992; Rammensee *et al.*, 1995). Although the sites of bond lysis within the protruding C-terminus are quite variable, the cleavages at the similarly-exposed N-terminus are very precise. This would appear to be a consequence of the number of proline residues within this sequence rendering it a poor substrate for aminopeptidases (Rötzschke & Falk, 1994).

Within the mouse CLIP sequence, proline residues are located at positions 82 and 87 (*Chapter 1*, Figure 1.5). The distance between these residues appears to be of a suitable length to permit endopeptidase cleavage between these two sites, most commonly between residues Ala 85 and Lys 86 (Rudensky *et al.*, 1991). This results in the majority of CLIP ligands eluted from mouse allotypes exhibiting Lys 86 as their N-terminal residue. It was for this reason, that the CLIP86–104 sequence was chosen to be the representative wild-type peptide in all the experiments described in this thesis. The protection of the N-terminus of the CLIP sequence from proteolysis results in these ligands being some of the longest seen binding to mouse class II MHC molecules — a minimum of 15 residues in length within the typical range of 12–19 amino acids (Rudensky *et al.*, 1991; Hunt *et al.*, 1992). Similarly, naturally-processed human CLIP ligands fall at the larger end of the range of peptides eluted from HLA molecules, in this case measuring at least 21 residues within a range of 10–28 amino acids. The extended

length of such human CLIP ligands relative to their mouse counterparts arises from the presence of two additional proline residues within human CLIP at positions 84 and 85 (Ser and Ala in the mouse sequence, respectively; *Chapter 1*, Figure 1.5). Thus, human CLIP does not display the same groove-proximal cleavage site as seen in mouse Ii, but is lysed typically N-terminal to Leu 81 or Lys 83 (Chicz et al., 1992, 1993, 1994; Riberdy *et al.*, 1992; Sette *et al.*, 1992a). Peptides exhibiting identical intact N-terminal sequence may also be generated *in vitro* by the proteolytic digestion of αβIi complexes (Ghosh *et al.*, 1995). However, amino-terminal proline residues are not unique to CLIP but are also found in a number of other class II MHC ligands where they likewise appear to function as stop signals for N-terminal trimming by aminopeptidases once the peptide has bound within the groove (Kropshofer *et al.*, 1993). Such a feature may be of use in predicting potential T-cell epitopes within a protein (Godkin *et al.*, 1998).

Mindful of the problems that exist in comparing different types of experimental data, a closer examination of the manner in which N-terminal CLIP residues are proposed to interact with distinct class II MHC molecules does nevertheless reveal a number of similarities. Firstly, Kropshofer *et al.* (1995b) have proposed that the N-terminal residues of human CLIP may contact an effector site on the HLA-DR αβ dimer, presumably very close to the peptide-binding groove. In support of this notion, the CLIP81–89 fragment is able to mediate release of the core sequence, CLIP90–105, from HLA-DR3 when provided in a *trans* arrangement. Moreover, a kinetic analysis of this interaction indicated that these two fragments were not competing for the same binding site (Kropshofer *et al.*, 1995a). Similarly, the Ii-4 ligand does not compete directly with the antigenic peptides for the binding groove but is proposed to promote the exchange of peptides from cell-surface I-E^d and I-E^k molecules at allosteric effector site (Adams & Humphreys, 1995; Adams *et al.*, 1997). The inability of Ii-4 to bind within the groove presumably arises as a result of it lacking the majority of CLIP residues identified in this study as being important for such an interaction, residues 90–99 (*Chapter 5*, §5.4.1). Accordingly, this ligand is not found associated naturally with class II MHC molecules (*e.g.* Rudensky *et al.*, 1991; Hunt *et al.*, 1992).

Secondly, Kropshofer *et al.* (1995b) have suggested that the significance of the Lys 83 and Lys 86 residues in the self-release of human CLIP from HLA-DR molecules may lie in the nature of their side chains. Positively-charged primary amines have also been shown to promote the dissociation of CLIP from HLA-DR αβ dimers (Avva & Cresswell, 1994). Similarly, the Lys 83 residue of mouse CLIP was found to influence the binding affinity for I-A^k and I-E^d molecules in this study (*Chapter 4*, Figures 4.10 & 4.11; *Chapter 5*, Figure 5.9) and mouse Ii-4 exhibits five positively-charged side chains,

one of which, Arg 82, is critical within the septameric minimal sequence, CLIP77–83, for promoting peptide exchange from I-E^k and I-E^d (Adams *et al.*, 1997). Other peptides that have multiple positive charges also exhibit a similar effect as Ii-4 upon I-E-bound determinants. For example, typical ligands of I-E^d molecules themselves exhibit a number of basic residues (*Chapter 5*, Table 5.4) and the addition of a second such peptide to an I-E^d-restricted antigen-presentation assay has been shown to induce the exchange of the first peptide within the MHC groove (Adorini *et al.*, 1989). This interchange appears to take place irrespective of whether the competitor is added simultaneously with the original antigenic peptide or up to 24 h later, suggesting that the free ligand has an influence on the kinetics of dissociation of the bound peptide. Free I-E^d-binding peptides in general are able to accelerate the decline in presentation capacity of antigen-pulsed APC (Pedrazzini *et al.*, 1991). The inclusion of peptides enriched in positively-charged side chains, such as the homopolymer, poly-L-lysine (14–19-mer) or the neuropeptide, dynorphin A (Dyn1–13, a 13-mer exhibiting 5 basic residues) in a binding assay with detergent-solubilised I-E^d and the antigenic hen egg lysozyme peptide, HEL107–116, results in enhanced binding of the HEL peptide (de Kroon & McConnell, 1993, 1994). The stimulation of I-E^d-restricted T cells specific for the HEL107–116 peptide is also increased in the presence of these non-stimulatory competitors, indicative of an expansion in the number of I-E^d molecules complexed with this ligand.

In light of the possible role of positive charges in the interaction of N-terminal CLIP residues with different class II MHC molecules, the effector site on the $\alpha\beta$ dimers may contain a number of negatively-charged groups. For HLA-DR molecules, the location of this site has been proposed to be on the α_1 domain of the molecule following the observation that the binding of CLIP81–105 and CLIP81–98 to HLA-DR1, DR2 and DR3 molecules is inhibited in the presence of the superantigen *Staphylococcus aureus* enterotoxin B (SEB; Kropshofer *et al.*, 1995a; Vogt *et al.*, 1995), known to bind exclusively to this domain outside of the groove (Jardetzky *et al.*, 1994). Similarly, inability of SEB to inhibit the association of CLIP92–105 and other groove-residing peptides, together with the competitive nature of the kinetics of the SEB/CLIP81–105 inhibition, would suggest that SEB and the N-terminal CLIP residues, 81–89, are competing for the same or overlapping binding sites on the HLA-DR molecules. Candidate negatively-charged residues on the HLA-DR α_1 domain are αAsp 17, αGlu 21, and αAsp 35 (Kropshofer *et al.*, 1995a), interestingly, all of which are conserved or conservatively-substituted in the equivalent domains of H-2A, H-2E, HLA-DP and -DQ molecules. Furthermore, just such a region of negative charge has been detected on the I-E^k $\alpha\beta$ dimer in proximity to the amino terminus of the bound peptide during fluorescence

energy transfer experiments to assess the charge-to-charge interactions with the moth cytochrome *c* peptide, mCyt*c*88–103 (Boniface *et al.*, 1993).

Meanwhile, Kropshofer *et al.* (1995b) have proposed that the N-terminal Pro 87 residue of CLIP may also modulate the dissociation rate of this ligand from different HLA-DR molecules. Similarly, a proline residue at position 82 within mouse Ii-4 is deemed to be important for promoting peptide exchange from I-E^k and I-E^d (Adams *et al.*, 1997) and the experiments presented herein also found Pro 87 to influence the binding affinity of the mouse CLIP ligand for both I-A^k and I-E^d molecules, albeit in different ways and at different pH (compare *Chapter 4*, Figures 4.10 & 4.11 and *Chapter 5*, Figure 5.9). The importance of such a residue may reflect that it has a critical role in stabilising a particular conformation. Molecular modelling of the proline-rich mouse Ii-4 peptide has shown that this sequence is likely to form a type II polyproline helix with the positively-charged side chains aligned on one face (Adams & Humphreys, 1995). Similarly, CD measurements of the quasi-palindromic human Ii peptide, Ii78–92, have revealed a measure of order consistent with a significant contribution from multiple, stabilised β-turns resulting from the four prolines and six positively-charged side chains within this sequence (LRMKLPKPPKPVSKMR; Lu *et al.*, 1990). Moreover, the Pro 87 side chain was defined in the crystal structure of CLIP complexed with HLA-DR3 even though this residue extends well beyond the peptide-binding groove into the solvent and does not participate in any direct bonding interactions with the residues of the αβ dimer (Ghosh *et al.*, 1995). In this sense, the visualisation of both the residues, Pro 87 and Val 88, at the relative positions P-4 and P-3, respectively, in the X-ray crystallographic analysis of CLIP–HLA-DR3 is unusual. In similar analyses of other peptide–class II MHC complexes, only as many as 13 residues of the bound ligand exhibit interpretable electron density, typically those corresponding to relative positions, P-2–P11 (Stern *et al.*, 1994; Fremont *et al.*, 1996, 1998b; Dessen *et al.*, 1997; Scott *et al.*, 1998).

A further key feature in the self-release of CLIP from different class II MHC molecules is proton concentration. Specifically, both Kropshofer *et al.* (1995a, 1995b) and Urban *et al.* (1994) have noted that this process takes place with HLA-DR variants nearly exclusively at low pH. Moreover, HLA-DR3 molecules expressed in HLA-DM° cell lines may be loaded with antigenic peptide in a weakly-acidic environment at 37°C, indicative of the CLIP ligand dissociating from the peptide-binding groove under these conditions (Riberdy *et al.*, 1992; Monji *et al.*, 1994). In this respect, this process differs from the ability of polycationic peptides to facilitate the release of peptides from I-E molecules. All experiments reporting this latter phenomenon were conducted at neutral pH (Adorini *et al.*, 1989; Pedrazzini *et al.*, 1991; de Kroon & McConnell, 1993, 1994;

Adams & Humphreys, 1995; Adams *et al.*, 1997). Complexes of the dynorphin peptide with I-E^d are less stable at pH 5.0 than at pH 7.0, such that the ability of this peptide to enhance the dissociation of HEL107–116 from these molecules is reduced at acidic pH (de Kroon & McConnell, 1993, 1994). However, peptide exchange from I-E molecules is more pronounced in live APC compared with those fixed in glutaraldehyde, suggesting that internal cellular activities do influence these interactions (Adorini *et al.*, 1989; Pedrazzini *et al.*, 1991). It is possible that an intracellular environment is created within the endocytic compartments that is enriched in the competitor peptide and pre-loaded class II MHC complexes, so as to increase the likelihood of exchange taking place. Alternatively, the fixative process in glutaraldehyde may simply lead to the peptides becoming cross-linked to their αβ dimers such that they are no longer able to dissociate.

The different pH conditions under which the N-terminal residues of CLIP may modulate the binding of this peptide to HLA-DR and I-E molecules has led to several contrasting mechanisms being proposed to account for these processes. Firstly, Kropshofer *et al.* (1995b) at one time suggested that the self-release of CLIP from HLA-DR molecules may occur *via* a conformational change induced in the αβ dimer when the N-terminal residues interact with the effector site. However, it is difficult to envisage how this process might be facilitated at low pH since the negatively-charged groups on the α_1 domain would be expected to be protonated under such conditions thereby abrogating the formation of any electrostatic contacts. Such a mechanism is more compatible with the ability of mouse Ii-4 to facilitate the dissociation of peptides from the I-E peptide-binding groove at neutral pH, as suggested by Adams & Humphreys (1995). Instead, Kropshofer *et al.* (1995a) have proposed that stabilising interactions form between the human N-terminal residues of CLIP and the HLA-DR αβ dimer at neutral pH but become compromised under conditions of high proton concentration. Consistent with this, the sequence element, Ii71–88, in the intact Ii protein has also been reported to contact class II MHC molecules at pH 7.4 and contribute to stabilising the αβIi trimer (Vogt *et al.*, 1995; Stumptner & Benaroch, 1997). Such effects are believed to be particularly important for those class II MHC variants with a low affinity for CLIP. Nevertheless, the same N-terminal residues of the human CLIP sequence do still appear to impose physical constraints upon the adjacent sequence within the groove. For example, HLA-DR1 molecules complexed with intact Ii do not exhibit stability in SDS detergent like those occupied only with the CLIP fragment unless the N-terminal residues, 82–89, within the Ii protein are deleted (Stumptner & Benaroch, 1997). This is similar to the effect of replacing the groove-bound CLIP sequence within intact Ii with known antigen-derived ligands (Stumptner & Benaroch, 1997; Barton & Rudensky, 1998).

An additional mechanism has also been proposed by which the dissociation of ligands bound to I-E $\alpha\beta$ dimers may be accelerated by peptides enriched in basic residues. This involves specifically those polycationic competitors that are able to bind in the peptide-binding groove themselves, *e.g.* Dyn1–13 but not Ii-4. In this process, the competitor peptide is suggested to bind stepwise in the individual pockets of the MHC binding groove as each is vacated by the existing ligand. Although seemingly unfavourable in terms of entropy, such a process would account for the faster off-rate of peptides by preventing the reassociation of the original peptide. This process is believed to involve predominantly I-E^d molecules as their groove exhibits a number of negatively-charged pockets (*Chapter 5*; Table 5.4). Further support for this mechanism is provided by experimental results with the antigenic HEL peptide and Dyn1–13 that indicate that these ligands exhibit similar affinities and share the same binding site on the I-E^d heterodimer (de Kroon & McConnell, 1993). An examination of the kinetics of the peptide displacement reaction is compatible with an intermediate of two peptides bound to the same heterodimer (de Kroon & McConnell, 1994). However, given the close proximity of the α_1 effector site to the peptide-binding groove, it is also possible the Dyn peptide simply overlaps with the groove to yield the results seen by de Kroon & McConnell (1993, 1994).

It is clear that further work is still needed to clarify the exact role of the N-terminal CLIP residues in modulating the binding affinity of this ligand for different class II MHC molecules. Unfortunately, the experiments performed in this study seeking evidence of this phenomenon in CLIP-bound mouse I-A and I-E molecules do not contribute much to resolving the confusion. For example, the binding of N-terminally-substituted CLIP to I-E^d was examined herein using only a truncated form of the Ii-4 ligand, missing one of the critical residues implicated in the mechanism, Arg 78 (Adams *et al.*, 1997). Moreover, these experiments were conducted at acidic pH whereas this process is reported to take place for I-E molecules at neutral pH (Adams & Humphreys, 1995) and the optimum pH at which to investigate the possibility of a CLIP self-release mechanism from I-A molecules is unknown. In order to address all of these concerns, a thorough examination of the dissociation rates of N-terminally-substituted CLIP analogues from the individual mouse class II MHC molecules under different pH conditions should be undertaken. Of further significance, the enzyme-linked immunoassays conducted in this study to assess the binding affinity of N-terminally-substituted mouse CLIP for both I-A and I-E molecules used an inappropriate detergent in the citrate–phosphate binding buffer (*Appendix B5.3*). This detergent, IGEPAL CA-630, is similar in structure and chemical properties to the more well-known non-ionic detergent, Nonidet P-40 (NP-40), which has been shown to reduce the rate of dissociation of CLIP from HLA-DR3 and thereby

obscure any indications of a CLIP self-release process (Kropshofer *et al.*, 1995b). For example, in the presence of NP-40, the time of half-maximal dissociation, $t_{1/2}$, of CLIP81–104 from HLA-DR3 is nearly 6 hours at 37°C, pH 5.8, compared with ~30 minutes in the presence of the detergent Zwittergent-12 (ZW-12) or ~2 hours in a detergent-free system (Kropshofer *et al.*, 1995b). Similarly, Avva & Cresswell (1994) have shown that some detergents facilitate the dissociation of CLIP from HLA-DR molecules *in vitro* better than others. SDS, for example, not only induces CLIP dissociation but proceeds to denature the entire molecule. These researchers also found that detergents containing short (8–10 carbon), unbranched hydrocarbon chains, such as octyl glucoside, were also effective in facilitating CLIP removal. By contrast, little or no dissociation of CLIP was detected from HLA-DR in the presence of those with longer hydrocarbon chains, even at pH 5.0 where CLIP dissociation from these molecules is usually enhanced. For this reason, it would be advisable to perform future experiments with purified class II MHC αβ dimers in either ZW-12 or octyl glucoside or, alternatively, using a detergent-free system with water-soluble proteins similar to that employed by Urban *et al.* (1994).

In the event that a self-release mechanism is confirmed to operate upon mouse CLIP–class II MHC complexes, it then remains to be determined the extent to which such a process is significant *in vivo*. Certainly, some sort of release mechanism additional to that mediated by HLA-DM would appear to be responsible for the ability of $I\text{-}A^k$ molecules to bind antigenic peptides in HLA-DM° cell lines (Brooks *et al.*, 1994; Stebbins *et al.*, 1996). However, it would seem unnecessary that such a mechanism should also operate upon CLIP-bound I-E molecules. These complexes appear already to be remarkably unstable at neutral pH as a result of inadequate anchor residues and would therefore be expected to dissociate readily at the cell surface (*Chapter 5*, §5.4.4). Nevertheless, it may be envisaged that CLIP and similar polycationic peptides might play a role *in vivo* in facilitating the release of other peptides from I-E molecules. Given that cognate peptides typically bind irreversibly to class II MHC molecules (Buus *et al.*, 1986; Roosnek *et al.*, 1988; Reay *et al.*, 1992), this may be a distinct mechanism to broaden the spectrum of antigenic determinants that may be presented to T cells independently of the actions of HLA-DM/H-2M. However, the role of these latter proteins is not simply to facilitate the dissociation of class II MHC-bound peptides. HLA-DM and H-2M are also significant in maintaining the peptide-binding groove of empty αβ dimers in a form receptive for binding new ligands (*Chapter 1*, §1.5.2). Is a two-peptide intermediate also able to perform this function?

It is interesting to note that several features of the proposed self-release mechanism of CLIP from HLA-DR molecules do show remarkable similarities to the manner in which the HLA-DM proteins are believed to facilitate the release of this ligand from class II MHC αβ dimers. Firstly, like HLA-DM (Kropshofer *et al.*, 1996; van Ham *et al.*, 1996; Weber *et al.*, 1996), the N-terminal residues of CLIP have been shown to be capable of accelerating the release of not just this peptide, but also a range of others that lack optimal anchor residues (Kropshofer *et al.*, 1995b). Secondly, CLIP self-release is favoured under conditions of low pH and non-ionic detergent (Kropshofer *et al.*, 1995a, 1995b), similar to the actions of HLA-DM (Sanderson *et al.*, 1996). For example, while no association between HLA-DR and HLA-DM is detected in NP-40, solubilisation of these molecules in the weaker, non-ionic detergent, digitonin, does permit their coprecipitation (Sanderson *et al.*, 1996). This encourages speculation that both of these mechanisms of CLIP dissociation from the class II MHC peptide-binding groove may act operate by a similar means, possibly even *via* the same effector site. Indeed, the CerCLIP.1 monoclonal antibody that is specific for the N-terminal residues of CLIP that protrude from the groove, has been shown to block HLA-DM-mediated release of CLIP from HLA-DR3 (Denzin & Cresswell, 1995). Nevertheless, these two mechanisms do not appear to act cooperatively since the N-terminal residues are not required for HLA-DM to facilitate CLIP removal, *e.g.* HLA-DM accelerates the dissociation rate of both CLIP81–104 and the N-terminally-truncated, CLIP89–101, from HLA-DR1 and DR3 molecules equally well (van Ham *et al.*, 1996; Weber *et al.*, 1996).

The advantages and limitations of the three experimental methods used in this study to assess the binding of CLIP to different mouse class II MHC αβ dimers have been discussed previously in *Chapter 3* (§3.4.1). A recurring issue throughout this thesis has been whether the behaviour of different class II MHC αβ dimers in the presence of SDS detergent reflects accurately the capacity of these molecules to form stable associations with peptide ligands. It is of particular concern that this property should be interpreted in this manner by so many investigators when its molecular basis is still in the process of being elucidated (*e.g.* Nelson *et al.*, 1996; Natarajan *et al.*, 1999b). Moreover, an inexact correlation has been reported to exist between the stability of some peptide–class II MHC complexes in SDS and their half-lives *in vivo* (Reizis *et al.*, 1997b) while the relevance of this property to the ability of such complexes to stimulate a T cell response remains unclear (Wu *et al.*, 1996). A number of potential problems with this practice have also been perceived that may influence the experimental outcome. For example, the cellular background in which the class II MHC molecules are expressed may affect the availability of certain subsets of peptides, as may different growth medium preparations and the procedures used between individual laboratories to isolate the class II MHC proteins.

Also of note, there would appear to be no standardised final concentration of the SDS detergent with which such experiments are conducted — available literature cites a range anywhere between 0.2% and 4% (Billing *et al.*, 1976; Springer *et al.*, 1977; Pious *et al.*, 1985; Stumptner & Benaroch, 1997).

The approach chosen in this study by which to dissect the interaction between CLIP and different mouse class II MHC molecules has been to assess the binding ability of an array of substituted and/or length-altered analogues of this peptide sequence. Such a procedure allows a given peptide to be analysed at the level of the individual constituent amino acids to determine which side chains are important for modulating the MHC binding affinity through contacts with the residues of the peptide-binding groove and which are oriented outwards from the molecule and thereby, in the case of antigen-derived ligands, are able to influence T-cell responses through interactions with the TCR. This approach is over-simplistic in the sense that it assumes that each individual residue contributes to the binding interaction independently of the others in the peptide sequence. However, this study has shown that, through the careful comparison of the results with data on the known peptide-binding preferences of the αβ dimer of interest and the molecular structure of this protein from X-ray crystallographic studies and homology models, it is possible to achieve a remarkably accurate assessment of the manner in which a peptide may bind. Indeed, the utility of this technique is demonstrated by the capacity to predict correctly the binding affinity of a monosubstituted ligand for a given class II MHC molecule, *e.g.* the association of the P96K and M99K ligands with I-E^d (*Chapter 5*, Figure 5.10). Moreover, the CLIP sequence itself provides an excellent tool for this process by virtue of its ability to bind to so many different class II MHC variants.

As a further illustration of how CLIP may be employed to probe the peptide-binding features of different class II MHC molecules, this ligand has been used in this study to provide important information on how the peculiar sequence at positions β56 and β57 in I-A^{g7} affects the ability of this αβ dimer to interact with peptides (*Chapter 6*). Together, these details suggest means by which the I-A^{g7} class II MHC molecule may contribute to the development of autoimmune diabetes in NOD mice. The first step of this process is the escape of self-reactive $CD4^+$ T cells from tolerance induction in the thymus by the aforementioned mechanisms (*Chapter 6*, §6.4.6). Next, in the periphery, these T cells may recognise unique β-cell autoantigen epitopes presented by I-A^{g7} and their affinity for self-peptide–MHC targets may be relatively high. In normal mouse strains, this would lead to these cells being tolerised. In NOD mice, however, the reduced stability, low cell-surface expression and altered conformational features of the I-A^{g7} heterodimer may jointly cause the overall avidity of the interaction to be reduced below the threshold of

immunotolerogenic functions, falling instead within the range in which effector responses are generated. Serial engagement of the peptide–I-A^{g7} complexes may then lead to the activation of these autoreactive T cells and they begin to proliferate and release cytokines of the Th1 phenotype. It is possible that the bias towards the production of pro-inflammatory mediators may also be attributable to the atypical properties of the I-A^{g7} molecule — the same factors that determine whether an effector response is to be mounted might also influence of what nature it will be, *i.e.* the affinity of a peptide for a given MHC molecule, the affinity of the TCR for this complex and the ligand concentration on the surface of the APC (Murray *et al.*, 1993, 1994, 1998; Kumar *et al.*, 1995; Pfeiffer *et al.*, 1995). Similarly, the time period over which T cells differentiate may also be critical in determining whether Th1 or Th2 cells predominate (Rogers & Croft, 1999). In this respect, it is of interest to note that activated β-cell-specific T cells in the diabetes-resistant NOD.PD transgenic mice typically release cytokines of the Th2 phenotype (Singer *et al.*, 1998).

The many potential consequences of the βPro56His and βAsp57Ser mutations within the I-A^{g7} molecule upon the functioning of the NOD immune system are in accordance with the H-2^{g7} MHC representing the most important genetic loci affecting the susceptibility of these animals to diabetes (Prochazka *et al.*, 1989; Wicker *et al.*, 1989). However, the effects of the defective functioning of the I-A^{g7} heterodimer are not limited only to β-cell autoreactivity in these animals — in addition to insulitis and diabetes, NOD mice exhibit an abundance of other autoimmune manifestations (Table 7.1). For example, a Sjögren's syndrome-like pathology may be present in these animals, arising from inflammation of the lacrimal gland and the submandibular and parotid tissues of the salivary glands (Miyagawa *et al.*, 1986; Goillot *et al.*, 1991; Humphreys-Beher *et al.*, 1994; Robinson *et al.*, 1998). Like autoimmune diabetes, this condition has been shown to be the outcome of an autoreactive Th1-type response, in this case targeting the 120 kDa cytoskeletal protein, α-fodrin (Yanagi *et al.*, 1998) — the same autoantigen identified to be critical in this condition in humans (Haneji *et al.*, 1997). Also, these animals may be induced to develop experimental autoimmune conditions, for example, systemic autoimmune rheumatic disease (ARD), a disorder resembling systemic lupus erythematosus (SLE) may be generated by immunisation with heat-killed *Mycobacterium bovis* , (bacillus Calmette-Guérin vaccine; BCG; Baxter *et al.*, 1994a, 1994b). It would seem to be highly improbable that this widespread autoreactivity in NOD mice could result solely from the capacity of the I-A^{g7} molecule to bind a unique repertoire of autoepitopes, as proposed in the determinant selection model of autoimmunity. Instead, these findings are more likely indicative that these animals suffer a substantial defect in the immunoregulatory mechanisms that are responsible for maintaining tolerance to self-proteins. It may be

Table 7.1 *Autoimmune conditions affecting NOD mice*

Autoimmune manifestation		Reference
Spontaneous:	insulitis/ diabetes	Makino *et al.*, 1980
	thyroiditis	Asamoto *et al.*, 1984
	parathyroiditis	Krug *et al.*, 1991
	sialadenitis	Miyagawa *et al.*, 1986
	inflammation of adrenal cortex/testes	Asamoto *et al.*, 1984
	antibodies to multiple autoantigens	Reddy *et al.*, 1988
	anti-nuclear autoantibodies	Humphreys-Beher *et al.*, 1993
	haemolytic anaemia in senescence	Baxter & Mandel, 1991
Inducible:	autoimmune rheumatic disease (ARD)	Baxter *et al.*, 1994
	experimental rheumatoid arthritis (ERA)	Kouskoff *et al.*, 1996
	experimental autoimmune encephalomyelitis (EAE)	Bernard *et al.*, 1997
	experimental autoimmune prostatitis (EAP)	Rivero *et al.*, 1998

speculated that this may also arise, at least in part, from the atypical properties of I-A^{g7}, for example, through the failure of this class II MHC molecule to select appropriate suppressor specificities in the thymus and/or by its biochemical inadequacies hindering the effective activation of regulatory T cells.

The absence of diabetes in transgenic NOD mice that express I-A^{g7} molecules modified at position β56 and/or β57, serves to illustrate the importance of these two particular β-chain positions *in vivo* with regards to developing β-cell autoimmunity (*Chapter 6*, §6.1.4; Lund *et al.*, 1990a; Quartey-Papafio *et al.*, 1995; Singer *et al.*, 1998). However, it is interesting to note that the degree of protection conferred upon these animals differs between the specific transgenes. For example, NOD.PD mice (I-$A\beta^{g7Pro56Asp57}$) are protected from diabetes and develop only minimal peri-insulitis (Singer *et al.*, 1998). Yet, these animals do still exhibit β-cell specific autoantibodies and lymphocytic infiltration of the submandibular salivary glands. The phenotype is similar in NOD mice expressing the I-$A\beta^{g7Pro56}$ transgene, the NOD.PRO mice (Lund *et al.*, 1990a). By contrast, NOD.ASP mice (I-$A\beta^{g7Asp57}$) are not completely protected from diabetes (an incidence of 12% in female mice at 30 weeks compared with 60% in age-matched controls) and exhibit considerable islet infiltration in addition to sialadenitis and anti-insulin autoantibodies (Quartey-Papafio *et al.*, 1995).

In the context of the 2-checkpoint model of autoimmune diabetes progression (André *et al.*, 1996; *Chapter 6*, §6.1.3) and the similar benign/malignant description of islet-associated autoimmunity (Gazda *et al.*, 1997; Dilts & Lafferty, 1999), it is apparent that the expression of the modified I-A^{g7} transgenes in all three cases is unable to prevent the release of autoaggressive thymocytes from the thymus in these animals (*Checkpoint 1*; benign autoimmunity). However, peripheral immunoregulatory mechanisms now appear to be in place to thwart the effector phase of autoreactivity which culminates in the destruction of the β cells (*Checkpoint 2*; malignant autoimmunity). The finding that these mechanisms appear to be more effective in the NOD.PD and NOD.PRO animals than those expressing the I-$A\beta^{g7Asp57}$ transgene is interesting, given the results presented in this study which determine that the βSer57Asp mutation in I-A^{g7} has many more striking effects upon the binding of a representative peptide, CLIP, than that of βHis56Pro (*Chapter 6*, §6.4.3–§6.4.5). It may be envisaged that the enhanced cell-surface peptide exchange by the I-$A^{g7Pro56}$ molecule contributes to superior functioning of the extrathymic regulatory processes present in these animals. An alternative mechanism may be that the particular spatial arrangement of atoms within the peptide-binding groove that results from the presence of a proline residue at position β56 is better able to be recognised by regulatory T cells. The possibility that conformational features around the β56 and/or

β57 residues may influence T cell interactions (*Chapter 6*, §6.4.6) is supported by the differences in cytokine profile produced between islet-reactive T cells from wild-type NOD and NOD.PD mice (Singer *et al.*, 1998). In this respect, it would have been interesting to examine in this study the capacity of CLIP to bind to the double revertant molecule, I-$A^{g7Pro56Asp57}$ to see how this compared with the single substituted forms. Unfortunately, however, none of the cell lines in existence known to express this molecule were able to be obtained by this laboratory. Similarly, speculation regarding the ability of the specific residues present at the positions β56 and β57 to modulate T cell effector responses must remain tentative at this stage since appropriately-restricted T cell hybridomas were not available during the course of this study to examine this concept further.

Other possible mechanisms of protection from diabetes afforded by the expression of the transgenes I-$A^{g7Pro56}$, I-$A^{g7Asp57}$ and I-$A^{g7Pro56Asp57}$ in NOD mice are suggested from studies involving the introduction of other non-NOD class II MHC molecules into these animals. In this respect, the inability of the modified I-A^{g7} molecules to bring about the deletion of diabetogenic T cells does not appear to be unusual — these latter investigations have determined that intrathymic deletion or anergy of autoreactive specificities is a relatively uncommon means by which introduced molecules bestow protection from autoimmune diabetes — or perhaps just rarely complete (see reviews by Baxter & Cooke, 1995; Böhme *et al.*, 1995; Slattery & Miller, 1996). For example, transgenic NOD mice expressing I-A^k (Miyazaki *et al.*, 1990; Slattery *et al.*, 1990), I-A^b (Nishimoto *et al.*, 1987), I-A^d (Singer *et al.*, 1993) or a functional I-E molecule (Lund *et al.*, 1990a) are protected from diabetes but still exhibit to some extent a range of signs indicating the presence of an autoreactive T cell repertoire, *e.g.* sialadenitis, various degrees of insulitis, the capacity to provoke diabetes in these animals by treatment with the diabetogenic agent, cyclophosphamide (CYP) and/or the ability to transfer diabetes to lymphocyte-deficient NOD recipients. Hanson *et al.* (1996) have also shown that the prevention of diabetogenesis by the expression of an I-E transgene requires the continued presence of this molecule on peripheral APC, a finding incompatible with the clonal deletion theory of transgene protection which would only require thymic expression during T cell ontogeny. Moreover, Parish *et al.* (1993a) found no evidence of the deletion of specific Vβ TCR elements in diabetes-resistant NOD mice expressing an I-E transgene and Reich *et al.* (1991) have retracted their earlier evidence for such a process (Reich *et al.*, 1989). Nevertheless, the negative selection of diabetogenic $CD4^+$ thymocytes by introduced class II MHC molecules has been demonstrated in NOD mice that express a β-cell reactive, I-A^{g7}-restricted, transgenic TCR (Schmidt *et al.*, 1997, 1999). This deletion appears to take place following engagement of the TCR transgene-expressing thymocytes and the

non-NOD class II MHC heterodimers on thymic bone-marrow-derived APC and is complete in the presence of I-A^b, H-2^k and H-2^{nbl}. By comparison, mice that express only I-A^k or I-A^s attain only partial deletion of the diabetogenic T cell clones and exhibit a correlating degree of insulitis.

The presence of insulitis but not overt diabetes in the majority of NOD mice that express transgenic class II MHC molecules is more consistent with peripheral mechanisms of protection. Such mechanisms include *a.)* immune deviation, whereby transgenic class II MHC molecules stimulate the production of regulatory Th2-type cytokines, as discussed above for the NOD.ASP, NOD.PRO and NOD.PD mice, *b.)* determinant stealing, involving competition for a diabetogenic epitope between the transgenic heterodimer and I-A^{g7} which prevents the formation of the complex required for disease induction (Böhme *et al.*, 1995), *c.)* a related theory known as determinant capture, whereby diabetogenic epitopes are unavailable for associating with I-A^{g7} because the transgenic molecule has bound to an adjacent epitope on the same peptide chain (Deng *et al.*, 1993), *d.)* determinant displacement, where high affinity peptides derived from the transgenic class II MHC molecule itself out-compete diabetogenic ligands for binding to I-A^{g7} (Kikutani & Makino, 1992) or *e.)* the positive selection of immunoregulatory TCR specificities, a mechanism involving both thymic and peripheral activities (Lühder *et al.*, 1998). From the results of the study presented herein, the mechanism of determinant stealing, in particular, would seem on first appearance unlikely given the number of differences in the peptide-binding motifs of different mouse class II MHC molecules *versus* I-A^{g7}, especially those of the I-E isotype with their preference for basic residues at the P9 position (compare *Chapter 5*, Table 5.4 with *Chapter 6*, Table 6.6). Indeed, Gregori *et al.* (1999) have characterised recently the peptide-binding motif of the NOD I-E^{g7} molecule, *i.e.* the $\alpha\beta$ heterodimer that may be produced between the endogenous NOD I-Eβ^{g7} polypeptide and that encoded by an I-Eα transgene, and have shown it be quite different from that of I-A^{g7}. It might be, however, that such differences are the key to the success of this mechanism if, as suggested previously, low affinity ligands of disease-associated class II MHC molecules are a significant part of the problem in the development of autoimmune disease, through their inability to trigger the deletion of autoreactive T cells (*Chapter 6*, §6.4.6).

Further elucidation of the specific mechanism(s) that act to protect different class II MHC-transgenic NOD mice from diabetes must await refinement of the systems used to generate and study such animals since it is clear from the findings to date that some of the effects observed are complications arising from such factors as the tissue distribution of transgene expression (Böhme *et al.*, 1990; Parish *et al.*, 1993b; Podolin *et al.*, 1993;

Pilström & Böhme, 1997), the manner in which the transgene is introduced into the NOD mouse (Nishimoto *et al.*, 1987; Lund *et al.*, 1990a) and the number of copies of this element present in these animals (Hanson *et al.*, 1996; Singer *et al.*, 1996). For example, high copy numbers of I-Aβ-encoding transgenes cause abnormal development of B cells such that any resulting protection from diabetes in NOD may arise more from a deficiency in the numbers of APC present or in autoantibody production than from a specific function of the transgene itself (Gilfillan *et al.*, 1990a, 1990b). The report that diabetes incidence may be reduced also by the introduction of the wild-type I-A^{g7} transgene itself serves to highlight further some of the problems that exist in assessing MHC-transgenic NOD mice (Wherrett *et al.*, 1997). Nevertheless, such studies in general have provided explanation for the observation that the incidence of diabetes in NOD mice is highest when I-A^{g7} is expressed in a homozygous state (Prochazka *et al.*, 1989; Wicker *et al.*, 1989).

Despite the potentially widespread immunological effects of the atypical amino acids at positions β56 and β57 within the I-A^{g7} molecule upon the NOD mouse, it is still clear that the expression of this heterodimer alone is not sufficient for diabetes development. For example, the expression of even the entire NOD MHC in congenic mouse strains of the B10 or B6 genetic background did not lead to the development of insulitis or diabetes in these animals (*Chapter 6*, §6.1.2; Wicker *et al.*, 1995). Similarly, Biozzi AB/H mice express a novel MHC haplotype, H-2^{dq1}, which includes I-A^{g7} but these animals do not develop spontaneous autoimmune diabetes (Liu *et al.*, 1993). Likewise, CTS mice share their class II MHC genes with NOD mice but do not become diabetic (Ikegami *et al.*, 1990b; Koide & Yoshida, 1990). Significantly, however, the expression of the CTS MHC on a NOD background is diabetogenic (Makino *et al.*, 1991). These findings all serve to illustrate the contribution of other genetic aspects of NOD mice in the induction of autoimmune disease. For example, Piganelli *et al.* (1998) have described a defect in the ability of NOD macrophages to mature and proliferate in response to various growth factors. This appears to arise from a reduced capacity of these APC to process and present antigen, possibly resulting from a deficiency in the intracellular levels of the thiol coenzyme, glutathione (GSH), which is important for reducing disulphide bonds to enable unfolding of the tertiary structure of protein antigens. Together with the biochemical defects of the I-A^{g7} molecule, the impaired ability of NOD macrophages to process and/or present autoantigens may be envisaged to reduce the ability to T cells to become sufficiently activated to induce peripheral tolerance and/or to activate immunoregulatory T cells. Furthermore, NOD T cells themselves may exhibit an intrinsic inability to attain the activation threshold required to induce peripheral tolerance or a regulatory Th2 response. For example, abnormalities have been noted in the ability of

NOD T cells to produce IL-2 and IL-4 upon TCR ligation, a defect leading to proliferative hyporesponsiveness (anergy) of regulatory Th2 cells (Zipris *et al.*, 1991b; Rapoport *et al.*, 1993a; Jaramillo *et al.*, 1994). In addition, the activation of regulatory T cells by APC in a syngeneic mixed lymphocyte reaction (SMLR) is deficient in NOD mice (Serreze & Leiter, 1988). From these results, it has been suggested that NOD T cells exhibit a defect in their TCR-mediated signal transduction along the PKC/Ras/MAPK pathway of activation (Rapoport *et al.*, 1993b). NOD mice also appear to have a deficiency in the number of NK T cells they possess, a cellular subset proposed to be a major source of the cytokine, IL-4, from which Th2 cells are induced to develop (Bach *et al.*, 1997a). All of these possible effects may be woven into the model outlined above of the specific effects of the I-A^{g7} heterodimer to obtain a more comprehensive view of how autoimmune disease develops in these animals.

The importance of the remainder of the NOD genetic background besides the H-2^{g7} genotype upon the development of autoimmunity has been illustrated clearly in studies with congenic NOD mice that express alternative MHC genes. Despite the lack of I-A^{g7}, these animals still display an assortment of autoimmune manifestations. For example, the congenic mouse, NOD.H-2^{b}, which derives its MHC from the C57BL/10 (B10) strain, develops extensive lymphocytic infiltration of the pancreas and submandibular glands, as well as exhibiting autoantibodies (Wicker *et al.*, 1992). Meanwhile, the I-A^{k} class II MHC molecule has been identified as permissive for the development of experimentally-induced thyroiditis (Vladutiu & Rose, 1971) and, accordingly, NOD.H-2^{k} (or H-2^{b4}) congenic mice exhibit spontaneous thyroiditis and anti-thyroglobulin autoantibodies with an incidence increased over that observed in wild-type NOD mice (19.6% *versus* 14.3% at one year, respectively; Wicker *et al.*, 1995; Damotte *et al.*, 1997). Interestingly, diabetes, if not insulitis, is for the most part not present in any of these NOD congenic strains, suggesting that I-A^{g7} has an additional role in influencing the tissue to be targeted for autoimmune attack. Nevertheless, the incidence of these other manifestations of autoimmunity in these animals is still low compared with that of autoimmune diabetes in the wild-type NOD animals — 19.6% spontaneous thyroiditis in the H-2^{k} congenic mice at one year (Damotte *et al.*, 1997) *versus* up to 100% insulitis in wild-type NOD mice at 30 weeks with 70–90% of females exhibiting overt autoimmune diabetes (Makino *et al.*, 1980). This demonstrates without a doubt that the effects of background genes in the NOD mouse are still very much secondary to the effects of the I-A^{g7} class II MHC molecule in predisposing these animals to autoimmune pathology.

The results of this study have demonstrated clearly that the mutations at positions β56 and β57 in the I-A^{g7} heterodimer have significant impact upon the presentation of peptides by

this particular class II MHC molecule. Moreover, from an examination of these effects, it has been possible to envisage how it may be that the identity of the amino acid residues at these two positions in the β-chain of the I-A^{g7} molecule contributes to the development of autoimmunity in NOD mice. Although the βPro56His mutation is unique to the mouse I-A^{g7} molecule, the loss of an aspartic residue at position β57 of class II MHC molecules is a polymorphism known to occur in other animals and there too it appears to predispose an individual to autoimmune disease. For example, βAsp 57-negative class II MHC variants are common in other rodent strains used routinely to model human autoimmune conditions, both spontaneous and induced (Table 7.2). Most significantly, however, the lack of an aspartic acid residue at position β57 is a major factor in determining the susceptibility of humans to IDDM (*Chapter 6*, §6.1.4; Todd *et al.*, 1987). Furthermore, as with NOD mice, this particular polymorphism is also associated with the development of many other human autoimmune disorders (Table 7.3). The apparent evolutionary maintenance of amino acid polymorphism at position β57 in these various mammalian species has been suggested to emphasise the functional significance of this residue in modulating immune responses (Olerup *et al.*, 1997).

Since its derivation (Makino *et al.*, 1980), the NOD mouse has been studied extensively in the hope that elucidation of the factors that cause autoimmune diabetes in this animal may lead to a means of treating and/or preventing IDDM in humans. What then are the implications of the results presented herein regarding the development of IDDM in humans? For the most part, the results obtained concerning the effects of the βAsp57Ser sequence motif in I-A^{g7} on peptide binding may be applied also to human class II MHC molecules that lack an aspartic acid residue at this position. For example, it would be expected that βAsp 57-negative class II HLA molecules would bind preferentially peptide ligands that exhibit acidic P9 residues, to provide compensatory positive binding energy for the loss of the stabilising β57–α76 salt bridge. Indeed, analysis of the peptide-binding motif of HLA-DQ8 would indicate this to be true (Kwok *et al.*, 1996a, 1996b; Godkin *et al.*, 1997; Oiso *et al.*, 1997). However, to date, no extensive examinations of the binding of CLIP to such HLA molecules have been conducted. In this respect, the findings of this study would predict that, in order to sustain an association with βAsp 57-negative human class II MHC molecules, the CLIP ligand would form a number of atypical interactions between the side chains of its C-terminal residues and those of the HLA peptide-binding groove.

The likelihood that additional bonds are required to maintain the interaction between human CLIP and βAsp 57-negative class II HLA molecules is consistent with reports that these molecules, like I-A^{g7}, are less stable than those that exhibit an aspartic acid at this

Table 7.2 *Rodent models of autoimmune disease associated with βAsp 57-negative class II MHC molecules.*

Animal	Class II MHC	β57	Autoimmune condition	Reference
Biozzi mouse	I-A^{z7}	serine	chronic relapsing EAE	Baker *et al.*, 1990
			rheumatoid arthritis	Bouvet *et al.*, 1990
Bio-Breeding rat	RT1.B^{u}	serine	IDDM	Nakhooda *et al.*, 1977
			spontaneous lymphocytic thyroiditis	Sternthal *et al.*, 1981
Lewis rat	RT1.B^{l}	serine	experimental autoimmune encephalomyelitis (EAE)	Yamamura *et al.*, 1986
			collagen-induced arthritis (CIA)	Griffiths *et al.*, 1981
			experimental autoimmune myasthenia gravis (EAMG)	Lindstrom, 1979
			experimental autoimmune uveoretinitis (EAU)	Wacker & Kalsow, 1973
			experimental autoimmune myocarditis	Kodama *et al.*, 1990
			experimental autoimmune neuritis (EAN)	Kadlubowski *et al.*, 1980
Buffalo rat	RT1.B^{b}	serine	experimental autoimmune encephalomyelitis (EAE)	Jones *et al.*, 1990
			experimental autoimmune thyroiditis (EAT)	Silverman & Rose, 1975

Table 7.3 *The association of βAsp 57-negative class II HLA alleles with human autoimmune disease.*

Allele	β57	Disease	Reference
*DQB1*0201*	alanine	IDDM	Thorsby, 1995
		coeliac disease	Sollid *et al.*, 1989
		systemic lupus erythematosus	Scofield *et al.*, 1994
		myasthenia gravis	Spurkland *et al.*, 1991
		autoimmune thyroid disease	Santamaria *et al.*, 1994
		dermatitis herpetiformis	Otley *et al.*, 1991
		NAITP	L'Abbe *et al.*, 1992
		Addison's disease	Partanen *et al.*, 1994
		Stiff-man syndrome	Pugliese *et al.*, 1993
		multiple sclerosis	Haegert *et al.*, 1993
*DQB1*0302*	alanine	IDDM	Thorsby, 1995
		coeliac disease	Mantovani *et al.*, 1993
		chronic idiopathic urticaria	O'Donnell *et al.*, 1999
		insulin autoimmune syndrome	Uchigata *et al.*, 1992
		atrophic autoimmune thyroiditis	Cho *et al.*, 1993
		rheumatoid arthritis	Angelini *et al.*, 1992
		Addison's disease	Boehm *et al.*, 1991
		multiple sclerosis	Haegert *et al.*, 1993
*DRB1*0405*	serine	IDDM	Caillat-Zucman *et al.*, 1992
		rheumatoid arthritis	Tan *et al.*, 1993
		Vogt-Koyanagi-Harada disease	Shindo *et al.*, 1994

[a]neonatal autoimmune thrombocytopenic purpura

position (Buckner *et al.*, 1996; Reizis *et al.*, 1997a). Therefore, it may be envisaged that the same mechanisms proposed in *Chapter 6* (§6.4.6) for I-A^{g7} might be applied to these human molecules to explain the frequency with which they are associated with the development of autoimmunity. Of particular interest in this regard is the recent publication of Nepom & Kwok (1998). These investigators have reviewed the current structural and functional knowledge of IDDM-associated HLA-DQ alleles and have outlined a number of immunological possibilities by which these molecules may contribute to the development of autoreactivity through inefficient molecular interactions and intercellular miscommunications. These suggestions are very much in accord with those proposed herein for I-A^{g7}, *i.e.* the defective positive and negative selection of TCR specificities in the thymus leading to a potentially autoreactive T-cell repertoire circulating in the periphery, the presentation of a unique range of peptide ligands possibly including diabetogenic epitopes derived from β cell autoantigens, the inappropriate activation of T cells recognising such determinants and, lastly, the dysregulation of the ensuing autoimmune response culminating in the selective destruction of the β-cell islet tissue.

The situation in humans does differ from NOD mice however, in that gene products of several other class II HLA loci are expressed simultaneously with the βAsp 57-negative variants in these individuals and that such molecules have the capacity to modify the risk of autoimmunity developing, *e.g.* the molecules, HLA-DR4 (DRA/DRB1*0406) and HLA-DQ6 (DQA1*0102/DQB1*0602), both confer protection from IDDM when expressed alongside HLA-DQ8 (*Chapter 6*, §6.1.1). The mechanisms proposed to account for this are the same as those outlined above to explain the ability of different class II MHC encoding transgenes expressed in NOD mice to protect these animals from diabetes and/or insulitis. Once again, thymic deletion of self-reactive TCR specificities in these individuals would appear to be the least likely possibility given that signs of immune activation to islet antigens are still evident in non-progressor HLA-DQ6/DQ8 heterozygotes (*i.e.* those which do not develop diabetes) in the form of autoantibodies and subclinical β-cell dysfunction (McCulloch *et al.*, 1990; Pugliese *et al.*, 1995; Gianani *et al.*, 1996). Instead, the mechanisms of determinant stealing or determinant capture appear to be favoured to account for the ability of HLA polymorphism to modulate the risk of diabetes development. For example, it has been proposed that a hierarchy of affinities for binding diabetes-associated epitopes exists for different HLA variants due to their varying peptide-binding motifs, such that these molecules compete for binding the same or overlapping determinants — an individual is susceptible to developing IDDM if the molecule present with the highest affinity for the diabetogenic ligand is one which lacks an aspartic acid at position β57 (Nepom, 1990). This suggestion is supported by the finding that the protective molecule, HLA-DRA/DRB5*0101, binds with high affinity an

epitope from the GAD autoantigen that overlaps the determinant presented by HLA-DQ8 with homology to the Coxsackie P2-C protein (Bach *et al.*, 1997b). Alternatively, it has been suggested that it is the differential capacity of these molecules to present tolerogenic peptides that is of significance (Sheehy, 1992; Undlien *et al.*, 1997). Either way, these proposals presuppose that protective HLA molecules bind ligands with higher affinity than IDDM-susceptibility variants to form more stable complexes. The finding that the protective molecule, HLA-DQA1*0102/DQB1*0602, shows remarkable stability in SDS detergent (Reizis *et al.*, 1997a; Ettinger *et al.*, 1998), in marked contrast to HLA-DQ8 (Buckner *et al.*, 1996; Reizis *et al.*, 1997a) has been interpreted to support this notion, yet half-life measurements of both complexes *in vivo* are very similar (Reizis *et al.*, 1997a), illustrating again the point that the resistance of dimers to SDS-induced dissociation may be reflective more of conformational stability than of peptide-binding affinity. Nevertheless, it is possible that the particular conformation associated with this phenomenon is better able to induce T cell tolerance or stimulate a Th2-type effector response to repress autoreactivity.

The findings from transfection studies with NOD mice, as described above, demonstrate clearly that it is by no means inevitable that benign autoimmunity in these animals should progress to the so-called malignant state whereupon the β cells are destroyed. Similarly in humans, IDDM is characterised by a long, asymptomatic but autoantibody-positive stage from which only certain individuals advance to hyperglycaemia and clinical diabetes (*e.g.* Gorsuch *et al.*, 1981; Palmer *et al.*, 1983; Srikanta *et al.*, 1985; Atkinson *et al.*, 1986; Bonifacio *et al.*, 1990; Riley *et al.*, 1990). This prodromal phase thus represents a significant opportunity in which to intervene in the autoimmune process with therapies. To date, a number of preventative strategies have been tested in NOD mice with relative success (Table 7.4; reviewed by Bowman *et al.*, 1994), often owing to the ability of these agents to divert anti-islet immune reactivity away from the Th1-type response of the β-cell destructive process towards that of an anti-inflammatory Th2 profile. A number of these strategies have also been trialed in human subjects at high risk of developing IDDM or in newly-diagnosed patients, *e.g.* BCG vaccination (Shehadeh *et al.*, 1994), immunosuppression by cyclosporin A (Stiller *et al.*, 1984; Dupre *et al.*, 1988), subcutaneous injection of insulin (Keller *et al.*, 1993) and oral administration of nicotinamide (Vague *et al.*, 1987). However, the utility of these different therapeutic interventions for IDDM has been limited by the number of undesirable side effects which arise as a result of the generally non-specific manner in which these treatments act or through the use of preparations containing much 'immuno-irrelevant' material.

By contrast, it is possible that the vaccination of susceptible individuals with distinct

Table 7.4 *Strategies for the prevention of diabetes in NOD mice.*

Factor administered		Reference
Immunosuppressive agents:	cyclosporin A	Mori *et al.*, 1986
	FK-506	Miyagawa *et al.*, 1990
Cytokines:	TNF-α	Satoh *et al.*, 1989
	IL-1α	Jacob *et al.*, 1990
	IL-2	Serreze *et al.*, 1989
Antibodies:	anti-CD3	Chatenoud *et al.*, 1994
	anti-CD4	Shizuru *et al.*, 1988
	anti-CD8	Wang *et al.*, 1996
	anti-IFN-γ, anti-TNF-α	Rabinovitch, 1994
	anti-IL-12	Rabinovitch, 1994
	anti-TCR	Sempe *et al.*, 1991
	anti-I-A	Boitard *et al.*, 1988
	anti-B7	Lenschow *et al.*, 1995
	anti-L-selectin	Yang *et al.*, 1993
	anti-VLA-4[a]	Yang *et al.*, 1993
Immunopotentiators:	BCG	Harada *et al.*, 1990
	CFA[b]	Sadelain *et al.*, 1990
	OK-432[c]	Toyota *et al.*, 1986
	microbial challenge	Singh & Rabinovitch, 1993
	corticosteroids	Rabinovitch *et al.*, 1992
	antioxidants	Rabinovitch *et al.*, 1992
Nicotinamide		Yamada *et al.*, 1982
Insulin:	oral	Zhang *et al.*, 1991
	subcutaneous	Atkinson *et al.*, 1990
Soluble TCR		McKeever *et al.*, 1996
Peptide antigens		*see text*

[a]very late antigen adhesion molecule 4
[b]complete Freund's adjuvant
[c]preparation of a low virulence *Streptococcus pyogenes* strain

peptide antigens could be a more selective approach by which the development of the autoimmune pathology might be prevented. In this respect, studies such as this advance considerably our ability to identify appropriate immunogenic determinants within whole proteins by contributing to an understanding of the manner in which different MHC molecules bind unique subsets of peptides. Through an appreciation of such details, it may then be possible to develop synthetic protein analogues that will be able to minimise adverse reactions while still maintaining high affinity for the MHC molecule and maximum immunogenicity. Knowledge of the molecular basis of different MHC peptide-binding motifs may also be used to induce the specific type of immune response desired, simply by altering the composition of a vaccine, for example, with regard to Th1 *versus* Th2-inducing determinants.

Regarding IDDM, it may be envisaged that the occupation of the class II MHC binding site by a peptide competitor may serve to prevent the presentation of diabetogenic determinants to autoaggressive T lymphocytes or alternatively, in the case of immunodominant epitopes, may induce a response which regulates such cells (reviewed by Adorini & Nagy, 1990a, 1990b; Smilek *et al.*, 1990; Fairchild *et al.*, 1994). An equivalent strategy has been shown to be effective in preventing the development of EAE in susceptible mouse strains (Wraith *et al.*, 1989; Gautam *et al.*, 1992a). More recently, a number of investigators have indeed demonstrated the capacity to inhibit the development of diabetes in NOD mice by immunisation with any of the peptides, p12(166–185) and p277(437–460) from mouse HSP60 (Elias & Cohen, 1995; Bockova *et al.*, 1997), residues 9–23 of the insulin B-chain (Daniel & Wegmann, 1996), p14(202–221) from GAD65 (Zechel *et al.*, 1997), a synthetic 14-mer (Hurtenbach *et al.*, 1993), λrep12–26 (Vaysburd *et al.*, 1995) and a cyclic peptide derived from residues β56–77 of I-A^{g7} itself (Dunsavage *et al.*, 1999). The data provided in this study aids in this process by contributing further information regarding the peptide-binding motif of the I-A^{g7} class II MHC molecule. In particular, this study has identified a CLIP analogue, M99E, which binds selectively and with high affinity to I-A^{g7}, but not to βAsp 57-positive mouse class II MHC molecules of the I-A isotype (compare *Chapter 6*, Figures 6.10 & 6.11). Given that βAsp 57-negative class II HLA molecules are also associated with susceptibility to IDDM in humans, it is possible that this information could be applied to peptide vaccine design for the prevention of human autoimmune diabetes as well.

One final question concerning the development of autoimmunity both in NOD mice and in humans is whether there exists any relevance of CLIP or invariant chain in this process. Given the critical functions of each of these components in the operation of the class II MHC-mediated pathway of antigen presentation (*Chapter 1*, §1.4.2 & §1.8), any

deviations in the affinity or timing of their interactions with the αβ dimer may be envisaged to have significant consequences upon the subsequent presentation of antigen to CD4$^+$ T lymphocytes, both in the thymus and the periphery. Indeed, the association of invariant chain and/or CLIP with class II MHC molecules to preclude the inappropriate binding of peptides from intracellular self-proteins may be an important mechanism towards suppressing autoimmune responses (*e.g.* Charron, 1992; Ansari, 1993). This notion is supported by the observation that susceptibility to collagen-induced arthritis is diminished in mice that exhibit impaired Ii degradation through a deficiency in the expression of the lysosomal cysteine proteinase, cathepsin S (Nakagawa *et al.*, 1999). With regards to NOD mice, the likelihood that the levels of CLIP associated with I-A^{g7} at the plasma membrane may be different from other mouse class II MHC molecules has been discussed previously, including the possibility that the resultant reduction in cell-surface ligand exchange may affect the diversity of TCR specificities selected in the thymus (*Chapter 6*, §6.4.5 & §6.4.6). Consistent with this notion, 15% of autoreactive T cells from wild-type NOD mice also recognise the I-A^{g7} heterodimer in the context of the T2 cell line which lacks expression of H-2M, such that these T cells would appear to be interacting with class II MHC molecules either occupied with CLIP or devoid of peptides altogether (Kanagawa *et al.*, 1998). Contrasting levels of CLIP or other Ii-derived peptides have also been found with particular class II HLA molecules that confer different degrees of risk of developing human autoimmune conditions, *e.g.* IDDM (Chicz *et al.*, 1994), juvenile dermatomyositis (Reed *et al.*, 1997), rheumatoid arthritis (Kirschmann *et al.*, 1995; Friede *et al.*, 1996). Moreover, the possible effects of variable interactions between Ii/CLIP and class II MHC molecules may not be limited to autoimmunity — T cell recognition of CLIP–class II MHC complexes has been linked to other immunological disease states, including cyclosporin-induced syngeneic and autologous graft-*versus*-host (GVH) reactions (Hess *et al.*, 1997a, 1997b, 1998; Chen *et al.*, 1998) and house dust mite allergies, this latter condition involving the CLIP ligand acting as a weak agonist to stimulate T cells reactive to an allergen-derived peptide that shows some sequence homology (Kristensen *et al.*, 1996). Molecular mimicry has also been proposed to take place between CLIP and a fragment of the retroviral p12 Gag polyprotein during mouse acquired immunodeficiency syndrome (MAIDS) which is caused by a defective mouse leukaemia virus (MuLV; Morse, 1996). Meanwhile, Hyoty *et al.* (1993) have identified an identical 7-amino acid residue stretch shared between Ii (residues 56–62) and the mumps virus nucleocapsid protein which is capable of inducing some immunological cross-reactivity between these two proteins. The association of Ii with class II MHC molecules has also been shown to affect the binding ability of some superantigens (Ericson *et al.*, 1994; Vogt *et al.*, 1995) and, lastly, altered levels of expression of Ii have been reported in a number of malignant cell types, suggesting likely

consequences upon the anti-tumour immune response (*e.g.* Elliott *et al.*, 1987b; Yao & Humphreys, 1988; Clements *et al.*, 1992; Veenstra *et al.*, 1993; Lu *et al.*, 1994; Saito *et al.*, 1997; Bosshart & Jarrett, 1998; Jiang *et al.*, 1999). All of these findings serve to illustrate the need to achieve a thorough understanding of CLIP and invariant chain and their interactions with class II MHC molecules.

In conclusion, investigations such as this into the basic mechanisms underlying the binding of peptides to different class II MHC molecules lead to a better understanding of the intricacies of antigen presentation and how amino acid sequence polymorphism in and around the peptide-binding groove may impact upon the generation of T cell-mediated immune responses. This information is invaluable in the development of effective immunotherapies, both for combating infectious microbial agents and in the management of autoimmune conditions where the barrier of self–non-self discrimination has been breached. With respect to the treatment or prevention of the autoimmune pathology, the ultimate goal is to have the ability to identify self-peptides that are involved in the disease process and to engineer synthetic antagonists for selective immunological intervention. Through its ability to bind promiscuously, the CLIP sequence of invariant chain represents a remarkable tool with which these aspects of class II MHC antigen presentation may be investigated, with the possibility also of using this ligand as the basic framework upon which selective antagonists may be constructed.

APPENDICES

APPENDIX A: REAGENTS AND SUPPLIERS

Reagent	Supplier
ABTS, 2, 2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)	Sigma Chemical Co., MO, USA
acetic acid, glacial	Ajax Chemicals, NSW, Australia
acrylamide/ bisacrylamide	National Diagnostics, GA, USA
antipain, hydrochloride	Sigma Chemical Co., MO, USA
aprotinin	Sigma Chemical Co., MO, USA
APS, ammonium persulphate	Ajax Chemicals, NSW, Australia
benzalkonium chloride	Sigma Chemical Co., MO, USA
borax, sodium tetraborate	Ajax Chemicals, NSW, Australia
boric acid	May & Baker, VIC, Australia
bromophenol blue	Sigma Chemical Co., MO, USA
BSA, bovine serum albumin	Armour Pharmaceuticals, Eastbourne, UK
citric acid, monohydrate	BDH Chemicals, VIC, Australia
DMSO, dimethyl sulphoxide	Sigma Chemical Co., MO, USA
EDTA, ethylenediamine tetraacetic acid	Ajax Chemicals, NSW, Australia
ethanol	Ajax Chemicals, NSW, Australia
FBS, foetal bovine serum	Trace Biosciences, NSW, Australia
FITC, fluorescein isothiocyanate	Pierce, IL, USA
formaldehyde, formalin	Sigma Chemical Co., MO, USA
Geneticin, G-418 sulphate	Gibco BRL, NY, USA
glutamine, L-	Gibco BRL, NY, USA
glycerol	Merck , VIC, Australia
glycine	Merck, VIC, Australia
HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid	Boehringer-Mannheim, Mannheim, Germany
hydrochloric acid	Ajax Chemicals, NSW, Australia
hydrogen peroxide	BDH Chemicals, VIC, Australia
IGEPAL CA-630	Sigma Chemical Co., MO, USA

Reagent	Supplier
leupeptin, trifluoroacetate salt	Sigma Chemical Co., MO, USA
2-ME, β-mercaptoethanol	BDH Chemicals, Dorset, UK
methanol	Merck, VIC, Australia
milk powder, non-fat	Bonlac Food Industries, VIC, Australia
MRA, mycoplasma removal agent	ICN Biomedicals, Tokyo, Japan
penicillin–streptomycin solution	Sigma Chemical Co., MO, USA
pepstatin A	Sigma Chemical Co., MO, USA
phenol red	Sigma Chemical Co., MO, USA
PI, propidium iodide	Sigma Chemical Co., MO, USA
PMSF	Sigma Chemical Co., MO, USA
potassium chloride	Ajax Chemicals, NSW, Australia
RPMI 1640 medium	Commonwealth Serum Labs, VIC, Australia
SDS, sodium dodecyl sulphate	BDH Chemicals, Dorset, UK
silver nitrate	Sigma Chemical Co., MO, USA
sodium acetate	Ajax Chemicals, NSW, Australia
sodium azide	BDH Chemicals, Dorset, UK
sodium bicarbonate	Sigma Chemical Co., MO, USA
sodium carbonate	Merck, VIC, Australia
sodium chloride	Merck, VIC, Australia
sodium citrate	BDH Chemicals, VIC, Australia
sodium hydroxide	BDH Chemicals, VIC, Australia
sodium phosphate, dibasic	Mallinckrodt, VIC, Australia
thymidine, [methyl-^{3}H], 5 mCi	Amersham, NSW, Australia
tricine	Sigma Chemical Co., MO, USA
trimethylamine	BDH Chemicals, Dorset, UK
Tris	Gibco BRL, NY, USA
trypan blue	Sigma Chemical Co., MO, USA
Tween-20	Sigma Chemical Co., MO, USA

APPENDIX B: BUFFERS, SOLUTIONS AND MEDIA

B1. Cell culture

RPMI 1640 culture medium

208.8 g RPMI powdered medium
40 g $NaHCO_3$
→ Dissolve in ddH_2O and make up volume to 20 L.
→ Filter sterilise (0.2 µm) and store at 4°C in the dark.

→ For complete growth medium, add to 500 mL RPMI 1640 solution:

5–10% (v/v) heat-inactivated FBS	25–50 mL stock
0.05 mM β-mercaptoethanol	0.5 mL of 50 mM stock
2 mM L-glutamine	10 mL of 100 mM stock
10 mM HEPES	2.5 mL of 2 M stock
antibiotics	1.25 mL Pen–Strep solution

→ Sterile filter (0.2 µm) into sterile Schott bottles and store at 4°C in the dark.

Heat-inactivated foetal bovine serum (FBS)

Incubate FBS in a water bath at 56°C for 2 hours.
Gently agitate solution periodically to ensure uniform heating.
Aliquot into sterile tubes and store at -20°C.

50 mM β-mercaptoethanol stock solution

β-mercaptoethanol (2-ME) stock concentration = 14.3 M.
Add 35 µL 2-ME to 10 mL complete RPMI media in fume hood.
Sterile filter (0.2 µm) and store at 4°C up to 4 months.

2 M HEPES

Dissolve 47.7 g in 100 mL ddH_2O.
→ Aliquot into 2.5 mL volumes and store at -20°C.

Mycoplasma Removal Agent

Mycoplasma Removal Agent (MRA) stock concentration = 50 µg/mL.
To treat cell culture for possible mycoplasma contamination, add MRA diluted to 0.5 µg/mL with complete RPMI medium.
Incubate for 1 week.

10 mg/mL G-418 sulphate stock solution

Dissolve 5 g Geneticin in 500 mL sterile complete RPMI medium.
Sterile filter (0.2 µm) and aliquot into 10 mL volumes.
Store at -20°C.

→ For 200 µg/mL solution: Add one 10 mL aliquot to 500 mL complete RPMI.

→ For 1 mg/mL solution: Add one 10 mL aliquot to 90 mL complete RPMI.

5% (w/v) Benzalkonium chloride stock solution

Dissolve 5 g of benzalkonium chloride in 100 mL ddH_2O.

→ Stir thoroughly to dissolve.

Humidity tray solution

0.025% (w/v) benzalkonium chloride	10 mL of 5% (w/v) stock
1% (w/v) SDS	200 mL of 10% (w/v) stock

→ Make up to 2 L with sterile ddH_2O.

Phosphate-buffered saline

1.9 mM NaH_2PO_4 (anhydrous)	0.23 g
8.1 mM Na_2HPO_4 (anhydrous)	1.15 g
154 mM NaCl	9.00 g

→ Dissolve in 900 mL ddH_2O and adjust pH to 7.4 with 1 M HCl.

→ Make up volume to 1 L and store at room temperature.

B2. Antibodies

B2.1 Protein A–Sepharose antibody purification

1.0 M Tris, pH 8.0

→ 24.23 g dissolved in 200 mL ddH_2O, adjust pH to 8.0 with concentrated HCl.

100 mM Tris, pH 8.0

→ Take 20 mL 1.0 M Tris, pH 8.0 and make up volume to 200 mL with ddH_2O.

10 mM Tris, pH 8.0

→ Take 2 mL 1.0 M Tris, pH 8.0 and make up volume to 200 mL with ddH_2O.

100 mM glycine, pH 3.0

→ 1.50 g dissolved in 200 mL ddH_2O, adjust pH to 3.0 with HCl.

B2.2 Biotinylation of monoclonal antibodies

Biotin labelling buffer

0.1 M $NaHCO_3$	16.80 g
0.1 M NaCl	11.69 g

→ Dissolve in ddH_2O and adjust pH to 8.4 with concentrated HCl.

→ Make up volume to 2 L and store at room temperature.

B2.3 FITC conjugation to monoclonal antibodies

FITC labelling buffer

0.05 M boric acid	6.18 g
0.2 M NaCl	23.38 g

→ Dissolve in ddH_2O and adjust pH from ≈6 to 9.2 with concentrated NaOH.

→ Make up volume to 2 L and store at 4°C.

→ Prepare buffer ≤2 weeks before use.

FITC dialysis buffer

0.1 M Tris	24.23 g
0.1% (w/v) NaN_3	20 mL of 10% (w/v) stock
0.2 M NaCl	23.38 g

→ Dissolve in ddH_2O and adjust pH from ≈11 to 7.4 with concentrated HCl.

→ Make up volume to 2 L and store at 4°C.

B3. Gel Electrophoresis

B3.1 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Resolving gel buffer, pH 8.8

90.855 g Tris dissolved in ddH_2O to 1.5 M

→ Adjust pH to 8.8 (from ≈12) with concentrated HCl.

→ Make up volume to 500 mL.

Stacking gel buffer, pH 6.8

24.228 g Tris dissolved in ddH_2O to 1.0 M

→ Adjust pH to 6.8 (from ≈11) with concentrated HCl.

→ Make up volume to 200 mL.

10% (w/v) Ammonium persulphate

Dissolve 0.5 g in 5 mL ddH_2O.

→ May be stored for several weeks at 4°C.

SDS-PAGE Tris-Glycine sample buffer (4×)

63 mM Tris, pH 6.8	2.5 mL 1 M stock
10% glycerol	4 mL
2% (w/v) SDS	0.8 g
0.0025% (w/v) bromophenol blue	200 µL of 0.5% (w/v)stock

→ Make up volume to 10 mL with ddH_2O.

→ For reducing conditions, add 2-ME to sample buffer to a final concentration of 2–5% (v/v) immediately before use.

SDS-PAGE Tris-Glycine running buffer (10×)

25 mM Tris	29 g
192 mM glycine	144 g
0.1% (w/v) SDS	10 g

→ Dissolve in ddH_20 and make up volume to 1 L.

→ 1× running buffer should be pH 8.3. Do not adjust with acid or base.

SDS-PAGE Tris-Tricine sample buffer (4×)

0.0625 M Tris, pH 6.8	2.5 mL of 2M stock
12% (v/v) glycerol	4.8 mL
2% (w/v) SDS	0.8 g
0.00125% (w/v) phenol red	500µL of 0.01% (w/v) stock

→ Make up volume to 10 mL with ddH_2O.

→ For reducing conditions, add 2-ME to 1× sample buffer to a final concentration of 2–5% (v/v) immediately before use.

SDS-PAGE Tris-Tricine anode buffer (10×)

0.2 M Tris	242.3 g

→ Dissolve in ddH_20 and adjust pH to 8.9.

→ Make up volume to 1 L.

SDS-PAGE Tris-Tricine cathode buffer (10×)

0.1 M Tris	121 g
0.1 M tricine	179 g
0.1% (w/v) SDS	10 g

→ Dissolve in ddH_20 and make up volume to 1 L.

→ 1× running buffer should be pH 8.3. Do not adjust with acid or base.

B3.2 Silver staining

Gel fixing solution

150 mL EtOH (analytical grade)

50 mL glacial acetic acid
300 mL ddH_2O

30% (v/v) EtOH
300 mL EtOH (analytical grade)
700 mL ddH_2O

20% (w/v) silver nitrate
Dissolve 1 g silver nitrate in 5 mL ddH_2O
Store in a brown bottle wrapped in foil
→ For a 0.1% (w/v) solution: 250 µL + 50 mL ddH_2O immediately before use.

2.5% (w/v) sodium carbonate/ 0.02% (w/w) formaldehyde
Dissolve 1.25 g sodium carbonate in 50 mL ddH_2O
Add 26.3 µL of a 38% (w/w) formalin solution (in fume hood)
→ Make fresh immediately before use.

1% (v/v) acetic acid
500 µL glacial acetic acid
50 mL ddH_2O

B3.3 Western blotting

NOVEX Western transfer buffer (10×)

12 mM Tris	29 g
96 mM glycine	144 g

→ Dissolve in ddH_2O and make up volume to 1.6 L.
→ pH should be ≈8.3. Do not adjust with acid or base.
→ Immediately before use dilute to 1× and add MeOH to 20% (v/v).

Western blocking buffer (PBS–T/ milk)

0.1% (v/v) Tween-20	500 µL
5% (w/v) non-fat milk powder	25 g

→ Dissolve in 500 mL cold PBS.

Western wash buffer (PBS–T)

0.1% (v/v) Tween-20	500 µL

→ Dissolve in 500 mL cold PBS.

B4. Purification of class II MHC proteins

B4.1 Preparation of antibody affinity columns

Coupling buffer

0.1 M $NaHCO_3$	16.8 g
0.5 M NaCl	58.4 g
0.02% (w/v) NaN_3	4 mL of 10% (w/v) stock

→ Dissolve in 1.5 L ddH_2O and adjust pH from ≈7.9 to 8.3 with NaOH.

→ Adjust volume to 2 L.

1 mM HCl

Add 43 µL concentrated HCl to 500 mL ddH_2O.

Alternatively dilute 500 µL 1 M HCl into 500 mL ddH_2O.

0.2 M Glycine buffer, pH 8.0

Dissolve 3 g glycine in 150 mL ddH_2O.

Adjust pH to 8.0 with NaOH and make up volume to 200 mL.

0.1 M Acetate buffer, pH 4.0

0.1 M NaOAc	1.64 g
0.5 M NaCl	5.844 g

→ Dissolve in 150 mL ddH_2O and adjust pH to 4.0.

→ Make up volume to 200 mL.

0.5% (v/v) IGEPAL/PBS

To 2 L sterile PBS, add 5 mL IGEPAL

→ Mix well and store at 4°C.

B4.2 Affinity purification of class II MHC molecules

PBS lysis buffer

Lyse cells at 10^8 *per* mL of 0.5% (v/v) IGEPAL/PBS

Immediately before use add protease inhibitors at the following recommended working concentrations:

2 mM EDTA
1 mM PMSF
2 µg/mL leupeptin
2 µg/mL antipain
2 µg/mL pepstatin A

2 μg/mL aprotinin

Class II MHC elution buffer

50 mM trimethylamine	0.7 mL (in fume hood)
150 mM NaCl	6 mL 5 M NaCl
2.5 mM NaN_3	0.2 mL 2.5 M NaN_3
5 mM EDTA	2 mL 0.5 M EDTA
10% (v/v) glycerol	20 mL glycerol
0.5% (v/v) IGEPAL	1 mL IGEPAL

→ Dissolve in ddH_2O, adjust pH to 11.5 and make up volume to 200 mL.

Citrate–phosphate neutralising buffer, pH 5.0

Stock solutions:

A: 0.1 M citric acid (monohydrate)	5.25 g in 250 mL ddH_2O
B: 0.2 M Na_2HPO_4 (anhydrous)	7.10 g in 250 mL ddH_2O

→ For pH 5.0 buffer: 24.3 mL A + 25.7 mL B, dilute to 100 mL with ddH_2O.

→ Add IGEPAL to 0.5% (v/v) and NaN_3 to 0.05% (w/v).

→ For eluate neutralisation: Add 80 μL into tubes for each mL to be collected.

Class II MHC dialysis buffer

0.5% (v/v) IGEPAL	10 mL
0.025% (w/v) NaN_3	10 mL of 10% (w/v) stock

→ Add to 2 L cold PBS.

B5. Peptide binding assays

B5.1 Antigen presentation assay

0.05 mCi/mL [^{3}H]thymidine

[^{3}H]thymidine stock concentration = 5 mCi/mL

Add 500 μL [^{3}H]thymidine to 10 mL complete RPMI media.

Sterile filter (0.2 μm) and store at 4°C.

→ For pulsing cells: dispense 10 μL per well (0.5 μCi).

B5.2 Cell-surface binding assay

FACS medium

1% (w/v) BSA	20 g
0.05% (w/v) NaN_3	10 mL of 10% (w/v) stock

→ Dissolve in 2 L cold PBS, stirring well.

→ Sterile filter (0.2 μm) and store at 4°C.

→ Alternative: add 20 mL heat-inactivated FBS (to 1% v/v) instead of BSA.

→ Note: The addition of sodium azide to the medium serves to slow cellular metabolism to minimise the modulation and internalisation of surface antigens due to antibody capping.

10 mg/mL Propidium iodide stock solution

Reconstitute 10 mg PI in 1 mL sterile PBS containing 0.1% (w/v) NaN_3.
Before use, titrate new stock against cell aliquots using serial dilutions in FACS medium from 100–1 µg/mL.

B5.3 Purified class II MHC binding assay

50 µg/mL goat anti-mouse IgG

Goat anti-mouse IgG (H+L) stock concentration ≈ 2.4 mg/mL

→ For one ELISA plate (10 mL): 208 µL IgG + 9792 µL PBS.

→ Note: Due to an excess of IgG, antibody solutions may be collected following incubation and reused for coating subsequent plates.

Borate buffer, pH 8.0

Stock solutions:

A: 0.2 M boric acid	3.09 g in 250 mL ddH_2O
B: 0.05 M borax	4.77 g in 250 mL ddH_2O

→ For pH 8.0 buffer: 50 mL A + 4.9 mL B, dilute to 200 mL with ddH_2O.

Citrate–phosphate binding buffer

Stock solutions:

A: 0.1 M citric acid (monohydrate)	5.25 g in 250 mL ddH_2O
B: 0.2 M Na_2HPO_4 (anhydrous)	7.10 g in 250 mL ddH_2O

→ For pH 5.0 buffer: 24.3 mL A + 25.7 mL B, dilute to 100 mL with ddH_2O.

→ For pH 7.0 buffer: 6.5 mL A + 43.6 mL B, dilute to 100 mL with ddH_2O.

→ Add IGEPAL to 0.5% (v/v) and NaN_3 to 0.01% (w/v).

Milk Buffer

0.1% (v/v) Tween-20	100 µL
5% (w/v) skim milk	5 g
0.1% (w/v) BSA	1 mL of 10% (w/v) stock
0.1% (w/v) NaN_3	1 mL of 10% (w/v) stock

→ Dissolve in 98 mL PBS.

→ Store at 4°C.

MBN solution

To 9800 µL of milk buffer, add 200 µL 25% (v/v) IGEPAL.

Neutralising Buffer

300 mM Tris, pH 7.5	9083µL
3.5% (w/v) skim milk	0.35 g
0.7% (w/v) BSA	700 µL of 10% (w/v) stock
0.07% (w/v) NaN_3	70 µL of 10% (w/v) stock
0.07% (v/v) Tween-20	7 µL
0.35% (v/v) IGEPAL	140 µL of 25% (v/v) stock

ABTS developing solution

ABTS	0.11 g
citric acid	0.55 g
sodium citrate	0.71 g

- → Dissolve in 100 mL ddH_2O.
- → pH should be 4.5 ± 0.2. Do not adjust.
- → Store at 4°C in dark (wrap bottle in foil).
- → *Per* plate: immediately before use add 10 µL H_2O_2 to 10 mL ABTS solution.
- → Test 100 µL on 20 µL streptavidin–HRP solution before adding to plate.

REFERENCES

Abbas, A. K., Murphy, K. M. and Sher, A. (1996) Functional diversity of helper T lymphocytes. *Nature* **383:** 787–793.

Acha-Orbea, H. and McDevitt, H. O. (1987) The first external domain of the non-obese diabetic mouse class II I-A β-chain is unique. *Proc. Natl Acad. Sci. USA* **84:** 2435–2442.

Acha-Orbea, H., Mitchell, D. J., Timmermann, L., Wraith, D. C., Tausch, G. S., Waldor, M. K., Zamvil, S. S., McDevitt, H. O. and Steinman, L. (1988) Limited heterogeneity of T cell receptors from lymphocytes mediating autoimmune encephalomyelitis allows specific immune intervention. *Cell* **54:** 263–273.

Acha-Orbea, H., Finke, D., Attinger, A., Schmid, S., Wehrli, N., Vacheron, S., Xenarios, I., Scarpellino, L., Toellner, K. M., MacLennan, I. C., *et al.* (1999) Interplays between mouse mammary tumor virus and the cellular and humoral immune response. *Immunol. Rev.* **168:** 287–303.

Adams, S. and Humphreys, R. E. (1995) Invariant chain peptides enhancing or inhibiting the presentation of antigenic peptides by major histocompatibility complex class II molecules. *Eur. J. Immunol.* **25:** 1693–1702.

Adams, S., Albericio, F., Alsina, J., Smith, E. R. and Humphreys, R. E. (1997) Biological activity and therapeutic potential of homologs of an Ii peptide which regulates antigenic peptide binding to cell-surface MHC class II molecules. *Arzneim.-Forsch.* **47:** 1069–1077.

Adorini, L. and Nagy, Z. A. (1990a) Peptide competition for antigen presentation. *Immunol. Today* **11:** 21–24.

Adorini, L. and Nagy, Z. (1990b) Inhibition of T cell activation by MHC blockade. *Int .Rev. Immunol.* **6:** 23–35.

Adorini, L., Sette, A., Buus, S., Grey, H. M., Darsley, M., Lehmann, P. V., Doria, G., Nagy, Z. A. and Appella, E. (1988a) Interaction of an immunodominant epitope with Ia molecules in T-cell activation. *Proc. Natl Acad. Sci. USA* **85:** 5181–5185.

Adorini, L., Appella, E., Doria, G. and Nagy, Z. A. (1988b) Mechanisms influencing the immunodominance of T cell determinants. *J. Exp. Med.* **168:** 2091–2104.

Adorini, L., Appella, E., Doria, G., Cardinaux, F. and Nagy, Z. A. (1989) Competition for antigen presentation in living cells involves exchange of peptides bound by class II MHC molecules. *Nature* **342:** 800–803.

Adorini, L., Moreno, J., Momburg, F., Hämmerling, G. J., Guéry, J.-C., Valli, A. and Fuchs, S. (1991) Exogenous peptides compete for the presentation of endogenous antigens to major histocompatibility complex class II-restricted T cells. *J. Exp. Med.* **174:** 945–948.

Agrawal, B., Manickasundari, M., Fraga, E. and Singh, B. (1991) T cells that recognise peptide sequences of self MHC class II molecules exist in syngeneic mice. *J. Immunol.* **147:** 383–390.

Akashi, T., Nagafuchi, S., Anzai, K., Kondo, S., Kitamura, D., Wakana, S., Ono, J., Kikuchi, M., Niho, Y. and Watanabe, T. (1997) Direct evidence for the contribution of B cells to the progression of insulitis and the development of diabetes in non-obese diabetic mice. *Int. Immunol.* **9:** 1159–1164.

Alam, S. M., Davies, G. M., Lin, C. M., Zal, T., Nasholds, W., Jameson, S. C., Hogquist, K. A., Gascoigne, N. R. and Travers, P. J. (1999) Qualitative and quantitative differences in T cell receptor binding of agonist and antagonist ligands. *Immunity* **10:** 227–237.

Albanesi, C., Cavani, A. and Girolomoni, G. (1998) IFN-γ-stimulated human keratinocytes express the genes necessary for the production of peptide-loaded MHC class II molecules. *J. Invest. Dermatol.* **110:** 138–142.

Alexander, J., Sidney, J., Southwood, S., Ruppert, J., Oseroff, C., Maewai, A., Snoke, K., Serra, H. M., Kubo, R. T., Sette, A., *et al.* (1994) Development of high potency universal DR-restricted helper epitopes by modification of high affinity DR-blocking peptides. *Immunity* **1:** 751–761.

Allen, P. M. (1987) Antigen processing at the molecular level. *Immunol. Today* **8:** 270–273.

Allen, P. M. and Unanue, E. R. (1984) Differential requirements for antigen processing by macrophages for lysozyme-specific T cell hybridomas. *J. Immunol.* **132:** 1077–1079.

Allen, P. M., Matsueda, G. R., Evans, R. J., Dunbar, J. B. J., Marshall, G. R. and Unanue, E. R. (1987) Identification of the T-cell and Ia contact residues of a T-cell antigenic epitope. *Nature* **327:** 713–715.

Allison, J. P., Walker, L. E., Russell, W. A., Pellegrino, M. A., Ferrone, S., Reisfeld, R. A., Frelinger, J. A. and Silver, J. (1978) Murine Ia and human DR antigens: Homology of amino-terminal sequences. *Proc. Natl Acad. Sci. USA* **75:** 3953–3956.

Amigorena, S., Drake, J. R., Webster, P. and Mellman, I. (1994) Transient accumulation of new class II MHC molecules in a novel endocytic compartment in B lymphocytes. *Nature* **369:** 113–119.

Amigorena, S., Webster, P., Drake, J., Newcomb, J., Cresswell, P. and Mellman, I. (1995) Invariant chain cleavage and peptide loading in major histocompatibility complex class II vesicles. *J. Exp. Med.* **181:** 1729–1741.

Amor, S., O'Neill, J. K., Morris, M. M., Smith, R. M., Wraith, D. C., Groome, N., Travers, P. J. and Baker, D. (1996) Encephalitogenic epitopes of myelin basic protein, proteolipid protein, and myelin oligodendrocyte glycoprotein for experimental allergic encephalomyelitis induction in Biozzi ABH (H-2A^{q}) mice share an amino acid motif. *J. Immunol.* **156:** 3000–3008.

Anderson, M. S. and Miller, J. (1992) Invariant chain can function as a chaperone protein for class II major histocompatibility complex molecules. *Proc. Natl Acad. Sci. USA* **89:** 2282–2286.

Anderson, K. S. and Cresswell, P. (1994) A role for calnexin (IP90) in the assembly of class II MHC molecules. *EMBO J.* **13:** 675–682.

Anderson, H. A. and Roche, P. A. (1998) Phosphorylation regulates the delivery of MHC class II invariant chain complexes to antigen processing compartments. *J. Immunol.* **160:** 4850–4858.

Anderson, G., Owen, J. J., Moore, N. C. and Jenkinson, E. J. (1994) Thymic epithelial cells provide unique signals for positive selection of $CD4^{+}CD8^{+}$ thymocytes in vitro. *J. Exp. Med.* **179:** 2027–2031.

Anderson, G., Hare, K. J. and Jenkinson, E. J. (1999a) Positive selection of thymocytes: the long and winding road. *Immunol. Today* **20:** 463–468.

Anderson, H. A., Bergstralh, D. T., Kawamura, T., Blauvelt, A. and Roche, P. A. (1999b) Phosphorylation of the invariant chain by protein kinase C regulates MHC class II trafficking to antigen-processing compartments. *J. Immunol.* **163:** 5435–5443.

André, I., Gonzalez, A., Wang, B., Katz, J., Benoist, C. and Mathis, D. (1996) Checkpoints in the progression of autoimmune disease: lessons from diabetes models. *Proc. Natl Acad. Sci. USA* **93:** 2260–2263.

Angelini, G., Morozzi, G., Delfino, L., Pera, C., Falco, M., Marcolongo, R., Giannelli, S., Ratti, G., Ricci, S., Fanetti, G., *et al.* (1992) Analysis of HLA DP, DQ, and DR alleles in adult Italian rheumatoid arthritis patients. *Hum. Immunol.* **34:** 135–141.

Ansari, A. A. (1993) A possible role of the MHC-associated invariant chain in rheumatoid arthritis. *Semin. Arthritis Rheum.* **23:** 193–197.

Arunachalam, B. and Cresswell, P. (1995) Molecular requirements for the interaction of class II major histocompatibility complex molecules and invariant chain with calnexin. *J. Biol. Chem.* **270:** 2784–2790.

Arunachalam, B., Lamb, C. A. and Cresswell, P. (1994) Transport properties of free and MHC class II-associated oligomers containing different isoforms of human invariant chain. *Int. Immunol.* **6:** 439–451.

Arunachalam, B., Pan, M. and Cresswell, P. (1998) Intracellular formation and cell-surface expression of a complex of an intact lysosomal protein and MHC class II molecules. *J. Immunol.* **160:** 5797–5806.

Asamoto, H., Akazawa, Y., Tashiro, S., Oishi, M., Azuma, T., Koide, S., Sudo, K., Yokota, H. and Tochino, Y. (1984) Infiltration of lymphocytes in various organs of the NOD (non-obese diabetic) mouse. *J. Japan Diab. Soc.* **27:** 775–781.

Asayama, K., Kooy, N. W. and Burr, I. M. (1986) Effect of vitamin E deficiency and selenium deficiency on insulin secretory reserve and free radical scavenging systems islets: decrease of islet manganosuperoxide dismutase. *J. Lab. Clin. Med.* **107:** 459–464.

Ashton-Rickardt, P. G. and Tonegawa, S. (1994) A differential-avidity model for T-cell selection. *Immunol. Today* **15:** 362–366.

Ashton-Rickardt, P. G., Bandeira, A., Delaney, J. R., Van Kaer, L., Pircher, H. P., Zinkernagel, R. M. and Tonegawa, S. (1994) Evidence for a differential avidity model of T cell selection in the thymus. *Cell* **76:** 651–663.

Astoul, E., Lafage, M. and Lafon, M. (1996) Rabies superantigen as a Vβ T-dependent adjuvant. *J. Exp. Med.* **183:** 1623–1631.

Atkinson, M. A. (1997) Mechanisms underlying the loss of self tolerance in NOD mice. *Res. Immunol.* **148:** 301–306.

Atkinson, M. A., Maclaren, N. K., Riley, W. J., Winter, W. E., Fisk, D. D. and Spillar, R. P. (1986) Are insulin autoantibodies markers for insulin-dependent diabetes mellitus? *Diabetes* **35:** 894–898.

Atkinson, M. A., Maclaren, N. K. and Luchetta, R. (1990) Insulitis and diabetes in NOD mice reduced by prophylactic insulin therapy. *Diabetes* **39:** 933–937.

Atkinson, M. A., Kaufman, D. L., Campbell, L., Gibbs, K. A., Shah, S. C., Bu, D. F., Erlander, M. G., Tobin, A. J. and Maclaren, N. K. (1992) Response of peripheral-blood mononuclear cells to glutamate decarboxylase in insulin-dependent diabetes. *Lancet* **339:** 458–459.

Atkinson, M. A., Bowman, M. A., Campbell, L., Darrow, B. L., Kaufman, D. L. and Maclaren, N. K. (1994) Cellular immunity to a determinant common to glutamate decarboxylase and Coxsackie virus in insulin-dependent diabetes. *J. Clin. Invest.* **94:** 2125–2129.

Avva, R. R. and Cresswell, P. (1994) *In vivo* and *in vitro* formation and dissociation of HLA-DR complexes with invariant chain-derived peptides. *Immunity* **1:** 763–774.

Awata, T., Kuzuya, T., Matsuda, A., Iwamoto, Y., Kanazawa, Y., Okuyama, M. and Juji, T. (1990) High frequency of aspartic acid at position 57 of HLA-DQ β-chain in Japanese IDDM patients and nondiabetic subjects. *Diabetes* **39:** 266–269.

Ayane, M., Mengle-Gaw, L., McDevitt, H. O., Benoist, C. and Mathis, D. (1986) $E\alpha^a$ and $E\beta^c$ chain association: Where lies the anomaly? *J. Immunol.* **137:** 948–951.

Bach, J.-F. and Mathis, D., orgs. (1997) 70th Forum in Immunology: The NOD mouse. *Res. Immunol.* **148:** 281–370.

Bach, J. F., Chatenoud, L., Herbelin, A., Gombert, J. M. and Carnaud, C. (1997a) Autoimmune diabetes: how many steps for one disease? *Res. Immunol.* **148:** 332–338.

Bach, J. M., Otto, H., Nepom, G. T., Jung, G., Cohen, H., Timsit, J., Boitard, C. and van Endert, P. M. (1997b) High affinity presentation of an autoantigenic peptide in type I diabetes by an HLA class II protein encoded in a haplotype protecting from disease. *J. Autoimmun.* **10:** 375–386.

Baekkeskov, S., Aanstoot, H. J., Christgau, S., Reetz, A., Solimena, M., Cascalho, M., Folli, F., Richter-Olesen, H., DeCamilli, P. and Camilli, P. D. (1990) Identification of the 64K autoantigen in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase. *Nature* **347:** 151–156.

Baisch, J. M., Weeks, T., Giles, R., Hoover, M., Stastny, P. and Capra, J. D. (1990) Analysis of HLA-DQ genotypes and susceptibility in insulin-dependent diabetes mellitus. *N. Engl. J. Med.* **322:** 1836–1841.

Baker, D., O'Neill, J. K., Gschmeissner, S. E., Wilcox, C. E., Butter, C. and Turk, J. L. (1990) Induction of chronic relapsing experimental allergic encephalomyelitis in Biozzi mice. *J. Neuroimmunol.* **28:** 261–270.

Bakke, O. and Dobberstein, B. (1990) MHC class II-associated invariant chain contains a sorting signal for endosomal compartments. *Cell* **63:** 707–716.

Bangia, N. and Watts, T. H. (1995) Evidence for invariant chain 85–101 (CLIP) binding in the antigen binding site of MHC class II molecules. *Int. Immunol.* **7:** 1585–1591.

Baquerizo, H. and Rabinovitch, A. (1990) Interferon-gamma sensitises rat pancreatic islet cells to lysis by cytokines and cytotoxic cells. *J. Autoimmun.* **3** (Suppl 1)**:** 123–130.

Barois, N., Forquet, F. and Davoust, J. (1998) Actin microfilaments control the MHC class II antigen presentation pathway in B cells. *J. Cell Sci.* **111:** 1791–1800.

Bartnes, K., Leon, F., Briand, J. P., Travers, P. J. and Hannestad, K. (1997) A novel primary anchor extends the MHC class II $I\text{-}A^d$ binding motif to encompass nine amino acids. *Int. Immunol.* **9:** 1185–1193.

Barton, G. M. and Rudensky, A. Y. (1998) An altered invariant chain protein with an antigenic peptide in place of CLIP forms SDS-stable complexes with class II αβ dimers and facilitates highly efficient peptide loading. *Int. Immunol.* **10:** 1159–1165.

Barton, G. M. and Rudensky, A. Y. (1999) Requirement for diverse, low-abundance peptides in positive selection of T cells. *Science* **283:** 67–70.

Baum, H., Butler, P., Davies, H., Sternberg, M. J. E. and Burroughs, A. K. (1993) Autoimmune disease and molecular mimicry: an hypothesis. *Trends. Biochem. Sci.* **18:** 140–144.

Baxter, A. G. and Mandel, T. E. (1991) Haemolytic anaemia in non-obese diabetic mice. *Eur. J. Immunol.* **21:** 2051–2055.

Baxter, A. G. and Cooke, A. (1995) The genetics of the NOD mouse. *Diabetes Metab. Rev.* **11:** 315–335.

Baxter, A. G., Healey, D. and Cooke, A. (1994a) Mycobacteria precipitate autoimmune rheumatic disease in NOD mice *via* an adjuvant-like activity. *Scand. J. Immunol.* **39:** 602–606.

Baxter, A. G., Horsfall, A. C., Healey, D., Ozegbe, P., Day, S., Williams, D. G. and Cooke, A. (1994b) Mycobacteria precipitate an SLE-like syndrome in diabetes-prone NOD mice. *Immunology* **83:** 227–231.

Beeson, P. B. (1994) Age and sex associations of 40 autoimmune diseases. *Am. J. Med.* **96:** 457–462.

Bell, G. I., Horita, S. and Karam, J. H. (1984) A polymorphic locus near the human insulin gene is associated with insulin-dependent diabetes mellitus. *Diabetes* **33:** 176–183.

Benacerraf, B. (1981) Role of MHC gene products in immune regulation. *Science* **212:** 1229–1238.

Benaroch, P., Yilla, M., Raposo, G., Ito, K., Miwa, K., Geuze, H. J. and Ploegh, H. L. (1995) How MHC class II molecules reach the endocytic pathway. *EMBO J.* **14:** 37–49.

Bendelac, A., Carnaud, C., Boitard, C. and Bach, J. F. (1987) Syngeneic transfer of autoimmune diabetes from diabetic NOD mice to healthy neonates. Requirement for both L3T4$^+$ and Lyt-2$^+$ T cells. *J Exp Med* **166:** 823–832.

Bendtzen, K., Mandrup-Poulsen, T., Nerup, J., Nielsen, J. H., Dinarello, C. A. and Svenson, M. (1986) Cytotoxicity of human pI 7 interleukin-1 for pancreatic islets of Langerhans. *Science* **232:** 1545–1547.

Benoist, C. and Mathis, D. (1997) Cell death mediators in autoimmune diabetes — No shortage of suspects. *Cell* **89:** 1–3.

Benoist, C. and Mathis, D. (1998) Autoimmunity. The pathogen connection. *Nature* **394:** 227–228.

Benoist, C. O., Mathis, D. J., Kanter, M. R., Williams, V. E., II and McDevitt, H. O. (1983a) Regions of allelic hypervariability in the murine Aα immune response gene. *Cell* **34:** 169–177.

Benoist, C. O., Mathis, D. J., Kanter, M. R., Williams, V. E. I. and McDevitt, H. O. (1983b) The murine Ia α-chains, Eα and Aα, show a surprising degree of sequence homology. *Proc. Natl Acad. Sci. USA* **80:** 534–538.

Bergerot, I., Arreaza, G., Cameron, M., Chou, H. and Delovitch, T. L. (1997) Role of T-cell anergy and suppression in susceptibility to IDDM. *Res. Immunol.* **148:** 348–358.

Bernard, C. C., Johns, T. G., Slavin, A., Ichikawa, M., Ewing, C., Liu, J. and Bettadapura, J. (1997) Myelin oligodendrocyte glycoprotein: a novel candidate autoantigen in multiple sclerosis. *J. Mol. Med.* **75:** 77–88.

Bertolino, P., Staschewski, M., Trescol-Biemont, M. C., Freisewinkel, I. M., Schenck, K., Chretien, I., Forquet, F., Gerlier, D., Rabourdin-Combe, C. and Koch, N. (1995) Deletion of a C-terminal sequence of the class II-associated invariant chain abrogates invariant chains oligomer formation and class II antigen presentation. *J. Immunol.* **154:** 5620–5629.

Bevan, M. J. (1997) In thymic selection, peptide diversity gives and takes away. *Immunity* **7:** 175–178.

Bevec, T., Stoka, V., Pungercic, G., Dolenc, I. and Turk, V. (1996) Major histocompatibility complex class II-associated p41 invariant chain fragment is a strong inhibitor of lysosomal cathepsin L. *J. Exp. Med.* **183:** 1331–1338.

Bhattacharya, A., Dorf, M. E. and Springer, T. A. (1981) A shared alloantigenic determinant on Ia antigens encoded by the I-A and I-E subregions: Evidence for I region gene duplication. *J. Immunol.* **127:** 2488–2495.

Bhayani, H. and Paterson, Y. (1989) Analysis of peptide binding patterns in different major histocompatibility complex/ T cell receptor complexes using pigeon cytochrome *c*-specific T cell hybridomas. *J. Exp. Med.* **170:** 1609–1625.

Bikoff, E. K., Huang, L. Y., Episkopou, V., van Meerwijk, J., Germain, R. N. and Robertson, E. J. (1993) Defective major histocompatibility complex class II assembly, transport, peptide acquisition, and $CD4^+$ T cell selection in mice lacking invariant chain expression. *J. Exp. Med.* **177:** 1699–1712.

Biljmakers, M.-J. E., Benaroch, P. and Ploegh, H. L. (1994a) Mapping functional regions in the lumenal domain of the class II-associated invariant chain. *J. Exp. Med.* **180:** 623–629.

Biljmakers, M.-J. J. E., Benaroch, P. and Ploegh, H. L. (1994b) Assembly of HLA-DR1 molecules translated *in vitro*: binding of peptide in the endoplasmic reticulum precludes association with invariant chain. *EMBO J.* **13:** 2699–2707.

Billing, R. J., Safani, M. and Peterson, P. (1976) Isolation and characterisation of human B cell alloantigens. *J. Immunol.* **117:** 1589–1593.

Birk, O. S., Douek, D. C., Elias, D., Takacs, K., Dewchand, H., Gur, S. L., Walker, M. D., van der Zee, R., Cohen, I. R. and Altmann, D. M. (1996) A role of Hsp60 in autoimmune diabetes: analysis in a transgenic model. *Proc. Natl Acad. Sci. USA* **93:** 1032–1037.

Bjorkman, P. J., Saper, M. A., Samraoui, B., Bennett, W. S., Strominger, J. L. and Wiley, D. C. (1987a) The foreign antigen binding site and T cell recognition regions of class I histocompatibility antigens. *Nature* **329:** 512–518.

Bjorkman, P. J., Saper, M. A., Samraoui, B., Bennett, W. S., Strominger, J. L. and Wiley, D. C. (1987b) Structure of the human class I histocompatibility antigen, HLA-A2. *Nature* **329:** 506–512.

Blum, J. S. and Cresswell, P. (1988) Role for intracellular proteases in the processing and transport of class II HLA antigens. *Proc. Natl Acad. Sci. USA* **85:** 3975–3979.

Bockova, J., Elias, D. and Cohen, I. R. (1997) Treatment of NOD diabetes with a novel peptide of the hsp60 molecule induces Th2-type antibodies. *J. Autoimmunity* **10:** 323–329.

Bodansky, H. J., Grant, P. J., Dean, B. M., McNally, J., Bottazzo, G. F., Hambling, M. H. and Wales, J. K. (1986) Islet-cell antibodies and insulin autoantibodies in association with common viral infections. *Lancet* **2:** 1351–1353.

Bodmer, H., Viville, S., Benoist, C. and Mathis, D. (1994) Diversity of endogenous epitopes bound to MHC class II molecules limited by invariant chain. *Science* **263:** 1284–1286.

Boehm, B. O., Manfras, B., Seidl, S., Holzberger, G., Kuhnl, P., Rosak, C., Schoffling, K. and Trucco, M. (1991) The HLA-DQβ non-Asp-57 allele: a predictor of future insulin-dependent diabetes mellitus in patients with autoimmune Addison's disease. *Tissue Antigens* **37:** 130–132.

Boehncke, W. H., Takeshita, T., Pendleton, C. D., Houghten, R. A., Sadegh-Nasseri, S., Racioppi, L., Berzofsky, J. A. and Germain, R. N. (1993) The importance of dominant negative effects of amino acid side chain substitution in peptide-MHC molecule interactions and T cell recognition. *J. Immunol.* **150:** 331–341.

Bogen, B. and Lambris, J. D. (1989) Minimum length of an idiotypic peptide and a model for its binding to a major histocompatibility complex class II molecule. *EMBO J.* **8:** 1947–1952.

Bohach, G. A., Fast, D. J., Nelson, R. D. and Schlievert, P. M. (1990) Staphylococcal and streptococcal pyrogenic toxins involved in toxic shock syndrome and related illnesses. *Crit. Rev. Microbiol.* **17:** 251–272.

Böhme, J., Schuhbaur, B., Kanagawa, O., Benoist, C. and Mathis, D. (1990) MHC-linked protection from diabetes dissociated from clonal deletion of T cells. *Science* **249:** 293–295.

Böhme, J., Brenden, N., Rietz, C. and Pilström, B. (1995) Dominant disease protection in MHC-transgenic NOD mice. More that one mechanism? *The Immunologist* **3:** 45–50.

Boitard, C., Bendelac, A., Richard, M. F., Carnaud, C. and Bach, J. F. (1988) Prevention of diabetes in nonobese diabetic mice by anti-I-A monoclonal antibodies: transfer of protection by splenic T cells. *Proc. Natl Acad. Sci. USA* **85:** 9719–9723.

Boitard, C., Yasunami, R., Dardenne, M. and Bach, J. F. (1989) T cell-mediated inhibition of the transfer of autoimmune diabetes in NOD mice. *J. Exp. Med.* **169:** 1669–1680.

Boniface, J. J., Allbritton, N. L., Reay, P. A., Kantor, R. M., Stryer, L. and Davis, M. M. (1993) pH affects both the mechanism and the specificity of peptide binding to a class II major histocompatibility complex molecule. *Biochemistry* **32:** 11761–11768.

Boniface, J. J., Lyons, D. S., Wettstein, D. A., Allbritton, N. L. and Davis, M. M. (1996) Evidence for a conformational change in a class II major histocompatibility complex molecule occurring in the same pH range where antigen binding is enhanced. *J. Exp. Med.* **183:** 119–126.

Bonifacio, E., Bingley, P. J., Shattock, M., Dean, B. M., Dunger, D., Gale, E. A. and Bottazzo, G. F. (1990) Quantification of islet-cell antibodies and prediction of insulin-dependent diabetes. *Lancet* **335:** 147–149.

Bonnerot, C., Lankar, D., Hanau, D., Spehner, D., Davoust, J., Salamero, J. and Fridman, W. H. (1995) Role of B cell Igα and Igβ subunits in MHC class II-restricted antigen presentation. *Immunity* **3:** 335–347.

Bosshart, H. and Jarrett, R. F. (1998) Deficient major histocompatibility complex class II antigen presentation in a subset of Hodgkin's disease tumor cells. *Blood* **92:** 2252–2259.

Bot, A., Bot, S., Antohi, S., Karjalainen, K. and Bona, C. (1996) Kinetics of generation and persistence on membrane class II molecules of a viral peptide expressed on foreign and self proteins. *J. Immunol.* **157:** 3436–3442.

Bourdoulous, S., Durieu-Trautmann, O., Strosberg, A. D. and Couraud, P. O. (1993) Catecholamines stimulate MHC class I, class II and invariant chain gene expression in brain endothelium through different mechanisms. *J. Immunol.* **150:** 1486–1495.

Bouvet, J. P., Couderc, J., Bouthillier, Y., Franc, B., Ducailar, A. and Mouton, D. (1990) Spontaneous rheumatoid-like arthritis in a line of mice sensitive to collagen-induced arthritis. *Arthritis Rheum.* **33:** 1716–1722.

Bowlus, C. L., Ahn, J., Chu, T. and Gruen, J. R. (1999) Cloning of a novel MHC-encoded serine peptidase highly expressed by cortical epithelial cells of the thymus. *Cell. Immunol.* **196:** 80–86.

Bowman, M. A., Leiter, E. H. and Atkinson, M. A. (1994) Prevention of diabetes in the NOD mouse: implications for therapeutic intervention in human disease. *Immunol. Today* **15:** 115–120.

Braunstein, N. S., Germain, R. N., Loney, K. and Berkowitz, N. (1990) Structurally interdependent and independent regions of allelic polymorphism in class II MHC molecules. *J. Immunol.* **145:** 1635–1645.

Bremnes, B., Madsen, T., Gedde-Dahl, M. and Bakke, O. (1994) An LI and ML motif in the cytoplasmic tail of the MHC-associated invariant chain mediate rapid internalisation. *J. Cell Sci.* **107:** 2021–2032.

Brocke, S., Gaur, A., Piercy, C., Gautam, A., Gijbels, K., Fathman, C. G. and Steinman, L. (1993) Induction of relapsing paralysis in experimental autoimmune encephalomyelitis by bacterial superantigen. *Nature* **365:** 642–644.

Brocke, S., Hausmann, S., Steinman, L. and Wucherpfennig, K. W. (1998) Microbial peptides and superantigens in the pathogenesis of autoimmune diseases of the central nervous system. *Semin. Immunol.* **10:** 57–67.

Brodsky, F. M. and Guagliardi, L. E. (1991) The cell biology of antigen processing and presentation. *Annu. Rev. Immunol.* **9:** 707–744.

Bromme, D., Bonneau, P. R., Lachance, P., Wiederanders, B., Kirschke, H., Peters, C., Thomas, D. Y., Storer, A. C. and Vernet, T. (1993) Functional expression of human cathepsin S in *Saccharomyces cerevisiae*. Purification and characterisation of the recombinant enzyme. *J. Biol. Chem.* **268:** 4832–4838.

Brooks, A. G. and McCluskey, J. (1993) Class II-restricted presentation of a hen egg lysozyme determinant derived from endogenous antigen sequestered in the cytoplasm or endoplasmic reticulum of the antigen presenting cells. *J. Immunol.* **150:** 3690–3697.

Brooks, A., Hartley, S., Kjer-Nielsen, L., Perera, J., Goodnow, C. C., Basten, A. and McCluskey, J. (1991) Class II-restricted presentation of an endogenously-derived immunodominant T-cell determinant of hen egg lysozyme. *Proc. Natl Acad. Sci. USA* **88:** 3290–3294.

Brooks, A. G., Campbell, P. L., Reynolds, P., Gautam, A. M. and McCluskey, J. (1994) Antigen presentation and assembly by mouse I-A^k class II molecules in human APC containing deleted or mutated *HLA DM* genes. *J. Immunol.* **153:** 5382–5392.

Brown, J. H., Jardetzky, T. S., Saper, M. A., Samraoui, B., Bjorkman, P. J. and Wiley, D. C. (1988) A hypothetical model of the foreign antigen binding site of class II histocompatibility molecules. *Nature* **332:** 845–850.

Brown, J. H., Jardetzky, T. S., Gorga, J. C., Stern, L. J., Urban, R. G., Strominger, J. L. and Wiley, D. C. (1993) Three-dimensional structure of the human class II histocompatibility antigen HLA-DR1. *Nature* **364:** 33–39.

Buckner, J., Kwok, W. W., Nepom, B. and Nepom, G. T. (1996) Modulation of HLA-DQ binding properties by differences in class II dimer stability and pH-dependent peptide interactions. *J. Immunol.* **157:** 4940–4945.

Busch, R., Strang, G., Howland, K. and Rothbard, J. B. (1990) Degenerate binding of immunogenic peptides to HLA-DR proteins on B cell surfaces. *Int. Immunol.* **2:** 443–451.

Busch, R., Hill, C. M., Hayball, J. D., Lamb, J. R. and Rothbard, J. B. (1991) Effect of natural polymorphism at residue 86 of the HLA-DR β-chain on peptide binding. *J. Immunol.* **147:** 1292–1298.

Busch, R., Vturina, I. Y., Drexler, J., Momburg, F. and Hämmerling, G. J. (1995) Poor loading of major histocompatibility complex class II molecules with endogenously-synthesised short peptides in the absence of invariant chain. *Eur. J. Immunol.* **25:** 48–53.

Busch, R., Cloutier, I., Sekaly, R.-P. and Hämmerling, G. J. (1996) Invariant chain protects class II histocompatibility antigens from binding intact polypeptides in the endoplasmic reticulum. *EMBO J.* **15:** 418–428.

Busch, R., Reich, Z., Zaller, D. M., Sloan, V. and Mellins, E. D. (1998) Secondary structure composition and pH-dependent conformational changes of soluble recombinant HLA-DM. *J. Biol. Chem.* **273:** 27557–27564.

Buus, S., Sette, A., Colon, S. M., Jenis, D. M. and Grey, H. M. (1986) Isolation and characterisation of antigen-Ia complexes involved in T cell recognition. *Cell* **47:** 1071–1077.

Buus, S., Sette, A., Colon, S. M., Miles, C. and Grey, H. M. (1987) The relation between major histocompatibility complex (MHC) restriction and the capacity of Ia to bind immunogenic peptides. *Science* **235:** 1353–1358.

Buus, S., Sette, A., Colon, S. M. and Grey, H. M. (1988) Autologous peptides constitutively occupy the antigen binding site on Ia. *Science* **242:** 1045–1047.

Caillat-Zucman, S., Garchon, H. J., Timsit, J., Assan, R., Boitard, C., Djilali-Saiah, I., Bougneres, P. and Bach, J. F. (1992) Age-dependent HLA genetic heterogeneity of type 1 insulin-dependent diabetes mellitus. *J. Clin. Invest.* **90:** 2242–2250.

Calcinaro, F., Lafferty, K. J. and Shehadeh, N. N. (1996) Inflammatory mediators and development of autoimmune diabetes. *In:* Eisenbarth, G. S. and Lafferty, K. J. (*eds.*), Type I diabetes: Molecular, cellular and clinical immunology. Oxford University Press, New York.

Cameron, M. J., Meagher, C. and Delovitch, T. L. (1998) Failure in immune regulation begets IDDM in NOD mice. *Diabetes Metab. Rev.* **14:** 177–185.

Campbell, R. D. and Milner, C. M. (1993) MHC genes in autoimmunity. *Curr. Opin. Immunol.* **5:** 887–893.

Candéias, S., Katz, J., Benoist, C., Mathis, D. and Haskins, K. (1991) Islet-specific T-cell clones from nonobese diabetic mice express heterogeneous T-cell receptors. *Proc. Natl Acad. Sci. USA* **88:** 6167–6170.

Carbone, F. R. and Bevan, M. J. (1990) Class I-restricted processing and presentation of exogenous cell-associated antigen in vivo. *J. Exp. Med.* **171:** 377–387.

Carrasco-Marin, E., Shimizu, J., Kanagawa, O. and Unanue, E. R. (1996) The class II MHC I-A^{g7} molecules from non-obese diabetic mice are poor peptide binders. *J. Immunol.* **156:** 450–458.

Carrasco-Marin, E., Kanagawa, O. and Unanue, E. R. (1997) Insights into the chemistry and biology of the I-A^{g7} class II molecule. *Res. Immunol.* **148:** 291–301.

Carrington, M., Yeager, M. and Mann, D. (1993) Characterisation of HLA-DMB polymorphism. *Immunogenetics* **38:** 446–449.

Castaño, L. and Eisenbarth, G. S. (1990) Type I diabetes: A chronic autoimmune disease of human, mouse, and rat. *Annu. Rev. Immunol.* **8:** 647–679.

Castellino, F. and Germain, R. N. (1995) Extensive trafficking of MHC class II-invariant chain complexes in the endocytic pathway and appearance of peptide-loaded class II in multiple compartments. *Immunity* **2:** 73–88.

Catálfamo, M., Serradell, L., Roura-Mir, C., Kolkowski, E., Sospedra, M., Vives-Pi, M., Vargas-Nieto, F., Pujol-Borrell, R. and Jaraquemada, D. (1999) HLA-DM and invariant chain are expressed by thyroid follicular cells, enabling the expression of compact DR molecules. *Int. Immunol.* **11:** 269–277.

Cavarelli, J., Prevost, G., Bourguet, W., Moulinier, L., Chevrier, B., Delagoutte, B., Bilwes, A., Mourey, L., Rifai, S., Piemont, Y., *et al.* (1997) The structure of Staphylococcus aureus epidermolytic toxin A, an atypic serine protease, at 1.7 Å resolution. *Structure* **5:** 813–824.

Ceppellini, R., Frumento, G., Ferrara, G. B., Tosi, R., Chersi, A. and Pernis, B. (1989) Binding of labelled influenza matrix peptide to HLA-DR in living B lymphoid cells. *Nature* **339:** 392–394.

Cerundolo, V., Elliott, T., Elvin, J., Bastin, J. and Townsend, A. (1992) Association of the human invariant chain with H-2 D^b class I molecules. *Eur. J. Immunol.* **22:** 2243–2248.

Chang, C. H. and Flavell, R. A. (1995) Class II transactivator regulates the expression of multiple genes involved in antigen presentation. *J. Exp. Med.* **181:** 765–767.

Chao, C. C. and McDevitt, H. O. (1997) Identification of immunogenic epitopes of GAD 65 presented by A^{g7} in non-obese diabetic mice. *Immunogenetics* **46:** 29–34.

Chao, N. J., Timmerman, L., McDevitt, H. O. and Jacob, C. O. (1989) Molecular characterisation of MHC class II antigens (β_1 domain) in the BB diabetes-prone and -resistant rat. *Immunogenetics* **29:** 231–234.

Chapman, H. A. (1998) Endosomal proteolysis and MHC class II function. *Curr. Opin. Immunol.* **10:** 93–102.

Charlton, B., Taylor-Edwards, C., Tisch, R. and Fathman, C. G. (1994) Prevention of diabetes and insulitis by neonatal intrathymic islet administration in NOD mice. *J. Autoimmun.* **7:** 549–560.

Charron, D. J. (1992) New aspects of HLA: perspectives for rheumatoid arthritis. *Clin. Exp. Rheumatol.* **10:** 293–296.

Chatenoud, L., Thervet, E., Primo, J. and Bach, J. F. (1994) Anti-CD3 antibody induces long-term remission of overt autoimmunity in nonobese diabetic mice. *Proc. Natl Acad. Sci. USA* **91:** 123–127.

Chelvanayagam, G. (1997) A roadmap for HLA-DR peptide binding specificities. *Human Immunol.* **58:** 61–69.

Chen, W., Thoburn, C. and Hess, A. D. (1998) Characterisation of the pathogenic autoreactive T cells in cyclosporine-induced syngeneic graft-*versus*-host disease. *J. Immunol.* **161:** 7040–7046.

Cherry, R. J., Wilson, K. M., Triantafilou, K., O'Toole, P., Morrison, I. E., Smith, P. R. and Fernandez, N. (1998) Detection of dimers of dimers of human leukocyte antigen (HLA)-DR on the surface of living cells by single-particle fluorescence imaging. *J. Cell Biol.* **140:** 71–79.

Chervonsky, A. V., Gordon, L. and Sant, A. J. (1994) A segment of the MHC class II β-chain plays a critical role in targeting class II molecules to the endocytic pathway. *Int. Immunol.* **6:** 973–982.

Chervonsky, A. V., Wang, Y., Wong, F. S., Visintin, I., Flavell, R. A., Janeway, C. A. J. and Matis, L. A. (1997) The role of Fas in autoimmune diabetes. *Cell* **89:** 17–24.

Chervonsky, A. V., Medzhitov, R. M., Denzin, L. K., Barlow, A. K., Rudensky, A. Y. and Janeway, C. A., Jr. (1998) Subtle conformational changes induced in major histocompatibility complex class II molecules by binding peptides. *Proc. Natl Acad. Sci. USA* **95:** 10094–10099.

Chiang, H. L., Terlecky, S. R., Plant, C. P. and Dice, J. F. (1989) A role for a 70-kilodalton heat shock protein in lysosomal degradation of intracellular proteins. *Science* **246:** 382–385.

Chicz, R. M., Urban, R. G., Lane, W. S., Gorga, J. C., Stern, L. J., Vignali, D. A. A. and Strominger, J. L. (1992) Predominant naturally-processed peptides bound to HLA-DR1 are derived from MHC-related molecules and are heterogeneous in size. *Nature* **358:** 764–768.

Chicz, R. M., Urban, R. G., Gorga, J. C., Vignali, D. A. A., Lane, W. S. and Strominger, J. L. (1993) Specificity and promiscuity among naturally-processed peptides bound to HLA-DR alleles. *J. Exp. Med* **178:** 27–47.

Chicz, R. M., Lane, W. S., Robinson, R. A., Trucco, M., Strominger, J. L. and Gorga, J. C. (1994) Self-peptides bound to the type I diabetes associated class II MHC molecules HLA-DQ1 and HLA-DQ8. *Int. Immunol.* **6:** 1639–1649.

Cho, S. G., Attaya, M. and Monaco, J. J. (1991) New class II-like genes in the murine MHC. *Nature* **353:** 573–576.

Cho, B. Y., Chung, J. H., Shong, Y. K., Chang, Y. B., Han, H., Lee, J. B., Lee, H. K. and Koh, C. S. (1993) A strong association between thyrotropin receptor-blocking antibody-positive atrophic autoimmune thyroiditis and HLA-DR8 and HLA-DQB1*0302 in Koreans. *J. Clin. Endocrinol. Metab.* **77:** 611–615.

Choi, E., McIntyre, K., Germain, R. N. and Seidman, J. G. (1983) Murine I-A β-chain polymorphism: nucleotide sequences of three allelic I-A β genes. *Science* **221:** 283–286.

Chu, Z.-T. E., Carswell-Crumpton, C., Cole, B. C. and Jones, P. P. (1994) The minimal polymorphism of class II Eα-chains is not due to the functional neutrality of mutations. *Immunogenetics* **40:** 9–20.

Claesson, L., Larhammer, D., Rask, L. and Peterson, P. A. (1983) cDNA clone for the human γ-chain of class II histocompatibility antigens and its implications for protein structure. *Proc. Natl Acad. Sci. USA* **80:** 7395–7399.

Claesson-Welsh, L., Barker, P. E., Larhammar, D., Rask, L., Ruddle, F. H. and Peterson, P. A. (1984) The gene encoding the human class II antigen-associated gamma chain is located on chromosome 5. *Immunogenetics* **20:** 89–93.

Clements, V. K., Baskar, S., Armstrong, T. D. and Ostrand-Rosenberg, S. (1992) Invariant chain alters the malignant phenotype of MHC class II$^+$ tumor cells. *J. Immunol.* **149:** 2391–2396.

Clements, J. L., Boerth, N. J., Lee, J. R. and Koretzky, G. A. (1999) Integration of T cell receptor-dependent signaling pathways by adapter proteins. *Annu. Rev. Immunol.* **17:** 89–108.

Cohen, I. R. (1997a) Questions about NOD mouse diabetes. *Res. Immunol.* **148:** 286–291.

Cohen, I. R. (1997b) The Th1/Th2 dichotomy, hsp60 autoimmunity, and type I diabetes. *Clin. Immunol. Immunopathol.* **84:** 103–106.

Coleman, D. L., Kuzava, J. E. and Leiter, E. H. (1990) Effect of diet on incidence of diabetes in nonobese diabetic mice. *Diabetes* **39:** 432–436.

Coligan, J. E., Kruisbeek, A. M., Margulies, D. H., Shevach, E. M. and Strober, W. (1994) Current Protocols in Immunology, John Wiley & Sons, New York.

Conrad, B., Weissmahr, R. N., Boni, J., Arcari, R., Schupbach, J. and Mach, B. (1997) A human endogenous retroviral superantigen as candidate autoimmune gene in type I diabetes. *Cell* **90:** 303–313.

Copier, J., Kleijmeer, M. J., Ponnambalam, S., Oorschot, V., Potter, P., Trowsdale, J. and Kelly, A. (1996) Targeting signal and subcellular compartments involved in the intracellular trafficking of HLA-DMB. *J. Immunol.* **157:** 1017–1027.

Copier, J., Potter, P., Sacks, S. H. and Kelly, A. P. (1998) Multiple signals regulate the intracellular trafficking of HLA-DM in B-lymphoblastoid cells. *Immunology* **93:** 505–510.

Corr, M., Slanetz, A. E., Boyd, L. F., Jelonek, M. T., Khilko, S., al-Ramadi, B. K., Kim, Y. S., Maher, S. E., Bothwell, A. L. and Margulies, D. H. (1994) T cell receptor-MHC class I peptide interactions: affinity, kinetics, and specificity. *Science* **265:** 946–949.

Corzo, D., Salazar, M., Granja, C. B. and Yunis, E. J. (1995) Advances in HLA genetics. *Exp. Clin. Immunogenet.* **12:** 156–170.

Cosgrove, D., Bodmer, H., Bogue, M., Benoist, C. and Mathis, D. (1992) Evaluation of the functional equivalence of major histocompatibility complex class II A and E complexes. *J. Exp. Med.* **176:** 629–634.

Cresswell, P. (1998) Proteases, processing and thymic selection. *Science* **280:** 394–395.

Cucca, F., Lampis, R., Frau, F., Macis, D., Angius, E., Masile, P., Chessa, M., Frongia, P., Silvetti, M., Cao, A., *et al.* (1995) The distribution of DR4 haplotypes in Sardinia suggests a primary association of type I diabetes with DRB1 and DQB1 loci. *Hum. Immunol.* **43:** 301–308.

Cuervo, A. M. and Dice, J. F. (1996) A receptor for the selective uptake and degradation of proteins by lysosomes. *Science* **273:** 501–503.

D'Alessio, D. J. (1992) A case-control study of group B Coxsackie virus immunoglobulin M antibody prevalence and HLA-DR antigens in newly diagnosed cases of insulin-dependent diabetes mellitus. *Am. J. Epidemiol.* **135:** 1331–1338.

Dahlén, E., Dawe, K., Ohlsson, L. and Hedlund, G. (1998) Dendritic cells and macrophages are the first and major producers of TNF-α in pancreatic islets in the nonobese diabetic mouse. *J. Immunol.* **160:** 3585–3593.

Damotte, D., Colomb, E., Cailleau, C., Brousse, N., Charreire, J. and Carnaud, C. (1997) Analysis of susceptibility of NOD mice to spontaneous and experimentally induced thyroiditis. *Eur. J. Immunol.* **27:** 2854–2862.

Daniel, D., Gill, R. G., Schloot, N. and Wegmann, D. (1995) Epitope specificity, cytokine production profile and diabetogenic activity of insulin-specific T cell clones isolated from NOD mice. *Eur. J. Immunol.* **25:** 1056–1062.

Daniel, D. and Wegmann, D. R. (1996) Protection of nonobese diabetic mice from diabetes by intranasal or subcutaneous administration of insulin peptide B-(9–23). *Proc. Natl Acad. Sci. USA* **93:** 956–960.

Das, H. K., Lawrence, S. K. and Weissman, S. M. (1983) Structure and nucleotide sequence of the heavy chain gene of HLA-DR. *Proc. Natl Acad. Sci. USA* **80:** 3543–3547.

Davidson, H. W., Reid, P. A., Lanzavecchia, A. and Watts, C. (1991) Processed antigen binds to newly synthesised MHC class II molecules in antigen-specific B lymphocytes. *Cell* **67:** 105–116.

Davis, M. M. and Bjorkman, P. J. (1988) T-cell antigen receptor genes and T-cell recognition. *Nature* **334:** 395–402.

Davis, C. B., Mitchell, D. J., Wraith, D. C., Todd, J. A., Zamvil, S. S., McDevitt, H. O., Steinman, L. and Jones, P. P. (1989) Polymorphic residues on the I-A β-chain modulate the stimulation of T cell clones specific for the N-terminal peptide of the autoantigen myelin basic protein. *J. Immunol.* **143:** 2083–2093.

Dawson, R. M. C., Elliott, D. C., Elliott, W. H. and Jones, K. M. (1986) Data for biochemical research, 3rd edn. Clarendon Press, Oxford.

Day, C. E. and Jones, P. P. (1983) The gene encoding the Ia antigen-associated invariant chain (Ii) is not linked to the *H-2* complex. *Nature* **302:** 157–159.

de Kroon, A. I. P. M. and McConnell, H. M. (1993) Enhancement of peptide antigen presentation by a second peptide. *Proc. Natl Acad. Sci. USA* **90:** 8797–8801.

de Kroon, A. I. P. M. and McConnell, H. M. (1994) Kinetics and specificity of peptide–MHC class II complex displacement reactions. *J. Immunol.* **152:** 609–619.

De Maria, R. and Testi, R. (1998) Fas–FasL interactions: a common pathogenetic mechanism in organ-specific autoimmunity. *Immunol. Today* **19:** 121–125.

DeGrado, W. F. (1988) Design of peptides and proteins. *Adv. Prot. Chem.* **39:** 51–123.

Delaney, C. A., Pavlovic, D., Hoorens, A., Pipeleers, D. G. and Eizirik, D. L. (1997) Cytokines induce deoxyribonucleic acid strand breaks and apoptosis in human pancreatic islet cells. *Endocrinology* **138:** 2610–2614.

Demotz, S. (1993) DR αβ dimers released from complexes with invariant chain fail to stimulate alloreactive T cell clones. *Eur. J. Immunol.* **23:** 2100–2108.

Demotz, S., Grey, H. M. and Sette, A. (1990) The minimal number of class II MHC-antigen complexes needed for T cell activation. *Science* **249:** 1028–1030.

Demotz, S., Barbey, C., Corradin, G., Amoroso, A. and Lanzavecchia, A. (1993) The set of naturally processed peptides displayed by DR molecules is tuned by polymorphism of residue 86. *Eur. J. Immunol.* **23:** 425–432.

Deng, H., Apple, R., Clare-Salzler, M., Trembleau, S., Mathis, D., Adorini, L. and Sercarz, E. (1993) Determinant capture as a possible mechanism of protection afforded by major histocompatibility complex class II molecules in autoimmune disease. *J. Exp. Med.* **178:** 1675–1680.

Denzin, L. K. and Cresswell, P. (1995) HLA-DM induces CLIP dissociation from MHC class II αβ dimers and facilitates peptide loading. *Cell* **82:** 155–165.

Denzin, L. K., Robbins, N. F., Carboy-Newcomb, C. and Cresswell, P. (1994) Assembly and intracellular transport of HLA-DM and correction of the class II antigen-processing defect in T2 cells. *Immunity* **1:** 595–606.

Denzin, L. K., Hammond, C. and Cresswell, P. (1996) HLA-DM interactions with intermediates in HLA-DR maturation and a role for HLA-DM in stabilizing empty HLA-DR molecules. *J. Exp. Med.* **184:** 2153–2165.

Denzin, L. K., Sant'Angelo, D. B., Hammond, C., Surman, M. J. and Cresswell, P. (1997) Negative regulation by HLA-DO of MHC class II-restricted antigen processing. *Science* **278:** 106–109.

Dessen, A., Lawrence, C. M., Cupo, S., Zaller, D. M. and Wiley, D. C. (1997) X-ray crystal structure of HLA-DR4 (DRA*0101, DRB1*0401) complexed with a peptide from human collagen II. *Immunity* **7:** 473–481.

Deussing, J., Roth, W., Saftig, P., Peters, C., Ploegh, H. L. and Villadangos, J. A. (1998) Cathepsins B and D are dispensable for major histocompatibility complex class II-mediated antigen presentation. *Proc. Natl Acad. Sci. USA* **95:** 4516–4521.

Dilts, S. M. and Lafferty, K. J. (1999) Autoimmune diabetes: The involvement of benign and malignant autoimmunity. *J. Autoimmun.* **12:** 229–232.

Donermeyer, D. L. and Allen, P. M. (1989) Binding to Ia protects an immunogenic peptide from proteolytic degradation. *J. Immunol.* **142:** 1063–1068.

Douek, D. and Altmann, D. M. (1997) HLA-DO is an intracellular class II molecule with distinctive thymic expression. *Int. Immunol.* **9:** 355–364.

Driessen, C., Bryant, R. A., Lennon-Dumenil, A. M., Villadangos, J. A., Bryant, P. W., Shi, G. P., Chapman, H. A. and Ploegh, H. L. (1999) Cathepsin S controls the trafficking and maturation of MHC class II molecules in dendritic cells. *J. Cell. Biol.* **147:** 775–790.

Driscoll, P. C., Altman, J. D., Boniface, J. J., Sakaguchi, K., Reay, P. A., Omichinski, J. G., Appella, E. and Davis, M. M. (1993) Two-dimensional nuclear magnetic resonance analysis of a labelled peptide bound to a class II major histocompatibility complex molecule. *J. Mol. Biol.* **232:** 342–350.

Dunn, W. A., Jr. (1990) Studies on the mechanisms of autophagy: formation of the autophagic vacuole. *J. Cell Biol.* **110:** 1923–1933.

Dunsavage, M. B., O'Leary C, J., Baumgart, T. D., Solvason, N., Howard, M., Lafferty, K., Deshpande, S. and Reich, E. P. (1999) A conformationally-constrained MHC class II I-A^{g7}-derived peptide protects NOD mice from the development of diabetes. *J. Autoimmun.* **12:** 233–242.

Dupre, J., Stiller, C. R., Gent, M., Donner, A., von Graffenried, B., Heinrichs, D., Jenner, M., Keown, P., Mahon, J., Martell, R., *et al.* (1988) Clinical trials of cyclosporin in IDDM. *Diabetes Care* **11** (Suppl 1)**:** 37–44.

Eastman, S., Deftos, M., DeRoos, P. C., Hsu, D.-H., Teyton, L., Braunstein, N. S., Hackett, C. J. and Rudensky, A. (1996) A study of complexes of class II invariant chain peptide: major histocompatibility complex class II molecules using a new complex-specific monoclonal antibody. *Eur. J. Immunol.* **26:** 385–393.

Eisenbarth, G. S. and Lafferty, K. J., eds. (1996) Type I diabetes: Molecular, cellular and clinical immunology. Oxford University Press, New York.

Elias, D. and Cohen, I. R. (1995) Treatment of autoimmune diabetes and insulitis in NOD mice with heat shock protein 60 peptide p277. *Diabetes* **44:** 1132–1138.

Elias, D., Markovits, D., Reshef, T., van der Zee, R. and Cohen, I. R. (1990) Induction and therapy of autoimmune diabetes in the non-obese diabetic (NOD/Lt) mouse by a 65-kDa heat shock protein. *Proc. Natl Acad. Sci. USA* **87:** 1576–1580.

Elias, D., Reshef, T., Birk, O. S., van der Zee, R., Walker, M. D. and Cohen, I. R. (1991) Vaccination against autoimmune mouse diabetes with a T-cell epitope of the human 65-kDa heat shock protein. *Proc. Natl Acad. Sci. USA* **88:** 3088–3091.

Elias, D., Meilin, A., Ablamunits, V., Birk, O. S., Carmi, P., Konen-Waisman, S. and Cohen, I. R. (1997) Hsp60 peptide therapy of NOD mouse diabetes induces a Th2 cytokine burst and downregulates autoimmunity to various β-cell antigens. *Diabetes* **46:** 758–764.

Elliott, W. L., Stille, C. J., Thomas, L. J. and Humphreys, R. E. (1987a) An hypothesis on the binding of an amphipathic, α-helical sequence in Ii to the desetope of class II antigens. *J. Immunol.* **138:** 2949–2952.

Elliott, W. L., Lu, S., Nguyen, Q., Reisert, P. S., Sairenji, T., Sorli, C. H., Stille, C. J., Thomas, L. J. and Humphreys, R. E. (1987b) Hyperexpressed hairy leukemic cell Ii might bind to the antigen- presenting site of class II MHC molecules. *Leukemia* **1:** 395–396.

Elliott, R. B., Reddy, S. N., Bibby, N. J. and Kida, K. (1988) Dietary prevention of diabetes in the non-obese diabetic mouse. *Diabetologia* **31:** 62–64.

Elliott, E. A., Drake, J. R., Amigorena, S., Elsemore, J., Webster, P., Mellman, I. and Flavell, R. A. (1994a) The invariant chain is required for intracellular transport and function of major histocompatibility complex class II molecules. *J. Exp. Med.* **179:** 681–694.

Elliott, J. F., Qin, H. Y., Bhatti, S., Smith, D. K., Singh, R. K., Dillon, T., Lauzon, J. and Singh, B. (1994b) Immunisation with the larger isoform of mouse glutamic acid decarboxylase (GAD67) prevents autoimmune diabetes in NOD mice. *Diabetes* **43:** 1494–1499.

Elliott, T., Cerundolo, V., Elvin, J. and Townsend, A. (1991) Peptide-induced conformational change of the class I heavy chain. *Nature* **351:** 402–406.

Engelman, D. M., Steitz, T. A. and Goldman, A. (1986) Identifying nonpolar transbilayer helices in amino acid sequences of membrane proteins. *Annu. Rev. Biophys. Biophys. Chem.* **15:** 321–353.

Ericson, M. L., Sundstrom, M., Sansom, D. M. and Charron, D. J. (1994) Mutually-exclusive binding of peptide and invariant chain to major histocompatibility complex class II antigens. *J. Biol. Chem.* **269:** 26531–26538.

Ernst, B. B., Surh, C. D. and Sprent, J. (1996) Bone marrow-derived cells fail to induce positive selection in thymus reaggregation cultures. *J. Exp. Med.* **183:** 1235–1240.

Ettinger, R. A., Liu, A. W., Nepom, G. T. and Kwok, W. W. (1998) Exceptional stability of the HLA-DQA1*0102/DQB1*0602 αβ protein dimer, the class II MHC molecule associated with protection from insulin-dependent diabetes mellitus. *J. Immunol.* **161:** 6439–6445.

Evavold, B. D., Williams, S. G., Hsu, B. L., Buus, S. and Allen, P. M. (1992) Complete dissection of the Hb(64–76) determinant using T helper 1, T helper 2 clones, and T cell hybridomas. *J. Immunol.* **148:** 347–353.

Fairchild, P. J., Wildgoose, R., Atherton, E., Webb, S. and Wraith, D. C. (1993) An autoantigenic T cell epitope forms unstable complexes with class II MHC: a novel route for escape from tolerance induction. *Int. Immunol.* **5:** 1151–1158.

Fairchild, P. J., Thorpe, C. J., Travers, P. J. and Wraith, D. C. (1994) Modulation of the immune response with T-cell epitopes: the ultimate goal for specific immunotherapy of autoimmune disease. *Immunology* **81:** 487–496.

Falcone, M., Lee, J., Patstone, G., Yeung, B. and Sarvetnick, N. (1998) B lymphocytes are crucial antigen-presenting cells in the pathogenic autoimmune response to GAD65 antigen in nonobese diabetic mice. *J. Immunol.* **161:** 1163–1168.

Falk, K., Rötzschke, O., Stevanovic, S., Jung, G. and Rammensee, H.-G. (1994) Pool sequencing of natural HLA-DR, DQ, and DP ligands reveals detailed peptide motifs, constraints of processing, and general rules. *Immunogenetics* **39:** 230–242.

Farr, A., DeRoos, P. C., Eastman, S. and Rudensky, A. Y. (1996) Differential expression of CLIP:MHC class II and conventional endogenous peptide–MHC class II complexes by thymic epithelial cells and peripheral antigen-presenting cells. *Eur. J. Immunol.* **26:** 3185–3193.

Fernandez-Borja, M., Verwoerd, D., Sanderson, F., Aerts, H., Trowsdale, J., Tulp, A. and Neefjes, J. (1996) HLA-DM and MHC class II molecules co-distribute with peptidase-containing lysosomal subcompartments. *Int. Immunol.* **8:** 625–640.

Fields, B. A., Malchiodi, E. L., Li, H., Ysern, X., Stauffacher, C. V., Schlievert, P. M., Karjalainen, K. and Mariuzza, R. A. (1996) Crystal structure of a T-cell receptor β-chain complexed with a superantigen. *Nature* **384:** 188–192.

Figueroa, F. and Klein, J. (1986) The evolution of MHC class II genes. *Immunol. Today* **7:** 78–81.

Fineschi, B., Arneson, L. S., Naujokas, M. F. and Miller, J. (1995) Proteolysis of major histocompatibility complex class II-associated invariant chain is regulated by the alternatively spliced gene product, p41. *Proc. Natl Acad. Sci. USA* **92:** 10257–10261.

Fineschi, B., Sakaguchi, K., Appella, E. and Miller, J. (1996) The proteolytic environment involved in MHC class II-restricted antigen presentation can be modulated by the p41 form of invariant chain. *J. Immunol.* **157:** 3211–3215.

Fling, S. P., Arp, B. and Pious, D. (1994) *HLA-DMA* and *-DMB* genes are both required for MHC class II/peptide complex formation in antigen-presenting cells. *Nature* **368:** 554–558.

Forquet, F., Barois, N., Machy, P., Trucy, J., Zimmermann, V. S., Leserman, L. and Davoust, J. (1999) Presentation of antigens internalised through the B cell receptor requires newly synthesised MHC class II molecules. *J. Immunol.* **162:** 3408–3416.

Fraser, J. D. (1989) High-affinity binding of staphylococcal enterotoxins A and B to HLA-DR. *Nature* **339:** 221–223.

Freisewinkel, I. M., Schenk, K. and Koch, N. (1993) The segment of invariant chain that is critical for association with major histocompatibility complex class II molecules contains the sequence of a peptide eluted from class II polypeptides. *Proc. Natl Acad. Sci. USA* **90:** 9703–9706.

Fremont, D. H., Matsumura, M., Stura, E. A., Peterson, P. A. and Wilson, I. A. (1992) Crystal structures of two viral peptides in complex with murine MHC class I H-2K^b. *Science* **257:** 919–927.

Fremont, D. H., Hendrickson, W. A., Marrack, P. and Kappler, J. (1996) Structures of an MHC class II molecule with covalently bound single peptides. *Science* **272:** 1001–1004.

Fremont, D. H., Crawford, F., Marrack, P., Hendrickson, W. A. and Kappler, J. (1998a) Crystal structure of mouse H2-M. *Immunity* **9:** 385–393.

Fremont, D. H., Monnaie, D., Nelson, C. A., Hendrickson, W. A. and Unanue, E. R. (1998b) Crystal structure of I-A^k in complex with a dominant epitope of lysozyme. *Immunity* **8:** 305–317.

Friede, T., Gnau, V., Jung, G., Keilholz, W., Stevanovic, S. and Rammensee, H. G. (1996) Natural ligand motifs of closely related HLA-DR4 molecules predict features of rheumatoid arthritis associated peptides. *Biochim. Biophys. Acta* **1316:** 85–101.

Fujii, S., Senju, S., Chen, Y.-Z., Ando, M., Matsushita, S. and Nishimura, Y. (1998) The CLIP-substituted invariant chain efficiently targets an antigenic peptide to HLA class II pathway in L cells. *Hum. Immunol.* **59:** 607–614.

Fujita, T., Yui, R., Kusumoto, Y., Serizawa, Y., Makino, S. and Tochino, Y. (1982) Lymphocytic insulitis in a 'non-obese diabetic (NOD)' strain of mice: An immunohistochemical and electron microscope investigation. *Biomed. Res.* **3:** 429–443.

Fung-Leung, W. P., Surh, C. D., Liljedahl, M., Pang, J., Leturcq, D., Peterson, P. A., Webb, S. R. and Karlsson, L. (1996) Antigen presentation and T cell development in H2-M-deficient mice. *Science* **271:** 1278–1281.

Garcia, K. C. and Teyton, L. (1998) T-cell receptor peptide-MHC interactions: biological lessons from structural studies. *Curr. Opin. Biotechnol.* **9:** 338–343.

Garcia, K. C., Teyton, L. and Wilson, I. A. (1999) Structural basis of T cell recognition. *Annu. Rev. Immunol.* **17:** 369–397.

Gautam, A. M., Pearson, C. I., Sinha, A. A., Smilek, D. E., Steinman, L. and McDevitt, H. O. (1992a) Inhibition of experimental autoimmune encephalomyelitis by a nonimmunogenic non-self peptide that binds to I-A^u. *J. Immunol.* **148:** 3049–3054.

Gautam, A. M., Pearson, C., Quinn, V., McDevitt, H. O. and Milburn, P. J. (1995) Binding of an invariant chain peptide, CLIP, to I-A major histocompatibility complex class II molecules. *Proc. Natl Acad. Sci. USA* **92:** 335–339.

Gautam, A. M., Yang, M., Milburn, P. J., Baker, R., Bhatnagar, A., McCluskey, J. and Boston, T. (1997) Identification of residues in the class II-associated Ii peptide (CLIP) region of invariant chain that affect efficiency of MHC class II- mediated antigen presentation in an allele-dependent manner. *J. Immunol.* **159:** 2782–2788.

Gazda, L. S., Charlton, B. and Lafferty, K. J. (1997) Diabetes results from a late change in the autoimmune response of NOD mice. *J. Autoimmun.* **10:** 261–270.

Geluk, A., van Meijgaarden, K. E., Drijfhout, J. W. and Ottenhoff, T. H. M. (1995) CLIP binds to HLA class II using methionine-based, allele-dependent motifs as well as allele-independent supermotifs. *Mol. Immunol.* **32:** 975–981.

Genuardi, M. and Saunders, G. F. (1988) Localisation of the HLA class II-associated invariant chain gene to human chromosome band 5q32. *Immunogenetics* **28:** 53–56.

Gerling, I. C., Serreze, D. V., Christianson, S. W. and Leiter, E. H. (1992) Intrathymic islet cell transplantation reduces β-cell autoimmunity and prevents diabetes in NOD/Lt mice. *Diabetes* **41:** 1672–1676.

Germain, R. N. and Hendrix, L. R. (1991) MHC class II structure, occupancy and surface expression determined by post-endoplasmic reticulum antigen binding. *Nature* **353:** 134–139.

Germain, R. N. and Rinker, A. G. J. (1993) Peptide binding inhibits protein aggregation of invariant-chain free class II dimers and promotes surface expresson of occupied molecules. *Nature* **363:** 725–728.

Germain, R. N. and Stefanova, I. (1999) The dynamics of T cell receptor signaling: complex orchestration and the key roles of tempo and cooperation. *Annu. Rev. Immunol.* **17:** 467–522.

Geuze, H. J. (1998) The role of endosomes and lysosomes in MHC class II functioning. *Immunol. Today* **19:** 282–287.

Ghosh, P., Amaya, M., Mellins, E. and Wiley, D. C. (1995) The structure of an intermediate in class II MHC maturation: CLIP bound to HLA-DR3. *Nature* **378:** 457–462.

Giacoletto, K. S., Sant, A. J., Bono, C., Gorka, J., O'Sullivan, D. M., Quaranta, V. and Schwartz, B. D. (1986) The human invariant chain is the core protein of the human class II-associated proteoglycan. *J. Exp. Med.* **164:** 1422–1439.

Gianani, R. and Eisenbarth, G. S. (1996) Humoral autoimmunity. *In:* Eisenbarth, G. S. and Lafferty, K. J. (*eds.*), Type I diabetes: Molecular, cellular and clinical immunology. Oxford University Press, New York.

Gianani, R., Verge, C. F., Moromisato-Gianani, R. I., Yu, L., Zhang, Y. J., Pugliese, A. and Eisenbarth, G. S. (1996) Limited loss of tolerance to islet autoantigens in ICA$^+$ first degree relatives of patients with type I diabetes expressing the HLA DQβ1*0602 allele. *J. Autoimmun.* **9:** 423–425.

Gilfillan, S., Aiso, S., Michie, S. A. and McDevitt, H. O. (1990a) The effect of excess β-chain synthesis on cell-surface expression of allele-mismatched class II heterodimers in vivo. *Proc. Natl Acad. Sci. USA* **87:** 7314–7318.

Gilfillan, S., Aiso, S., Michie, S. A. and McDevitt, H. O. (1990b) Immune deficiency due to high copy numbers of an $A\beta^k$ transgene. *Proc. Natl Acad. Sci. USA* **87:** 7319–7323.

Ginsberg-Fellner, F., Witt, M. E., Yagihashi, S., Dobersen, M. J., Taub, F., Fedun, B., McEvoy, R. C., Roman, S. H., Davies, R. G., Cooper, L. Z., *et al.* (1984) Congenital rubella syndrome as a model for type 1 (insulin-dependent) diabetes mellitus: increased prevalence of islet cell surface antibodies. *Diabetologia* **27** (Suppl): 87–89.

Glimcher, L. H. and Kara, C. J. (1992) Sequences and factors: A guide to MHC class II transcription. *Annu. Rev. Immunol.* **10:** 13–49.

Glimcher, L. H., McKean, D. J., Choi, E. and Seidman, J. G. (1985) Complex regulation of class II gene expression: Analysis with class II mutant cell lines. *J. Immunol.* **135:** 3542–3550.

Goding, J. W. (1983) Monoclonal antibodies: Principles and practice, Academic Press, London.

Godkin, A., Friede, T., Davenport, M., Stevanovic, S., Willis, A., Jewell, D., Hill, A. and Rammensee, H. G. (1997) Use of eluted peptide sequence data to identify the binding characteristics of peptides to the insulin-dependent diabetes susceptibility allele HLA-DQ8 (DQ 3.2). *Int. Immunol.* **9:** 905–911.

Godkin, A. J., Davenport, M. P., Willis, A., Jewell, D. P. and Hill, A. V. (1998) Use of complete eluted peptide sequence data from HLA-DR and -DQ molecules to predict T cell epitopes, and the

influence of the nonbinding terminal regions of ligands in epitope selection. *J. Immunol.* **161:** 850–858.

Goillot, E., Mutin, M. and Touraine, J. L. (1991) Sialadenitis in nonobese diabetic mice: transfer into syngeneic healthy neonates by splenic T lymphocytes. *Clin. Immunol. Immunopathol.* **59:** 462–473.

Goodman, S., Sawada, T., Barbosa, J. A., Cole, B., Pergolizzi, R., Silver, J., Mellins, E. and Chang, M. D. (1995) Mutational analysis of two DR α-residues involved in dimers of HLA-DR molecules. *J. Immunol.* **155:** 1210–1217.

Gorer, P. A. (1936) The detection of antigenic differences in mouse erythrocytes by the employment of immune sera. *Br. J. Exp. Pathol.* **17:** 42–50.

Gorer, P. A. (1937) The genetic and antigenic basis of tumour transplantation. *J. Pathol. Bacteriol.* **44:** 691–697.

Gorga, J. C., Horejsí, V., Johnson, D. R., Raghupathy, R. and Strominger, J. L. (1987) Purification and characterisation of class II histocompatibility antigens from a homozygous human B cell line. *J. Biol. Chem.* **262:** 16087–16094.

Gorsuch, A. N., Spencer, K. M., Lister, J., McNally, J. M., Dean, B. M., Bottazzo, G. F. and Cudworth, A. G. (1981) Evidence for a long prediabetic period in type I (insulin-dependent) diabetes mellitus. *Lancet* **2:** 1363–1365.

Gorvel, J. P., Escola, J. M., Stang, E. and Bakke, O. (1995) Invariant chain induces a delayed transport from early to late endosomes. *J. Biol. Chem.* **270:** 2741–2746.

Green, N. M. (1975) Avidin. *Adv. Protein Chem.* **29:** 85–133.

Green, J. M., DeMars, R., Xu, X. and Pierce, S. K. (1995) The intracellular transport of MHC class II molecules in the absence of HLA-DM. *J. Immunol.* **155:** 3759–3768.

Gregori, S., Trembleau, S., Penna, G., Gallazzi, F., Hammer, J., Papadopoulos, G. K. and Adorini, L. (1999) A peptide binding motif for I-E^{q7}, the MHC class II molecule that protects Eα-transgenic nonobese diabetic mice from autoimmune diabetes. *J. Immunol.* **162:** 6630–6640.

Griffin, J. P., Chu, R. and Harding, C. V. (1997) Early endosomes and a late endocytic compartment generate different peptide-class II MHC complexes via distinct processing mechanisms. *J. Immunol.* **158:** 1523–1532.

Griffiths, M. M., Eichwald, E. J., Martin, J. H., Smith, C. B. and DeWitt, C. W. (1981) Immunogenetic control of experimental type II collagen-induced arthritis. I. Susceptibility and resistance among inbred strains of rats. *Arthritis Rheum.* **24:** 781–789.

Grubin, C. E., Kovats, S., deRoos, P. and Rudensky, A. Y. (1997) Deficient positive selection of $CD4^+$ T cells in mice displaying altered repertoires of MHC class II-bound self-peptides. *Immunity* **7:** 197–208.

Guerder, S., Meyerhoff, J. and Flavell, R. (1994) The role of the T cell costimulator B7-1 in autoimmunity and the induction and maintenance of tolerance to peripheral antigen. *Immunity* **1:** 155–166.

Guerra, C. B., Busch, R., Doebele, R. C., Liu, W., Sawada, T., Kwok, W. W., Chang, M. D. and Mellins, E. D. (1998) Novel glycosylation of HLA-DRα disrupts antigen presentation without altering endosomal localisation. *J. Immunol.* **160:** 4289–4297.

Guillet, J.-G., Lai, M.-Z., Briner, T. J., Buus, S., Sette, A., Grey, H. M., Smith, J. A. and Gefter, M. L. (1987) Immunological self, nonself discrimination. *Science* **235:** 865–870.

Guncar, G., Pungercic, G., Klemencic, I., Turk, V. and Turk, D. (1999) Crystal structure of MHC class II-associated p41 Ii fragment bound to cathepsin L reveals the structural basis for differentiation between cathepsins L and S. *EMBO J.* **18:** 793–803.

Haegert, D. G., Muntoni, F., Murru, M. R., Costa, G., Francis, G. S. and Marrosu, M. G. (1993) HLA-DQA1 and -DQB1 associations with multiple sclerosis in Sardinia and French Canada: evidence for immunogenetically distinct patient groups. *Neurology* **43:** 548–552.

Hammer, J., Valsasnini, P., Tolba, K., Bolin, D., Higelin, J., Takacs, B. and Sinigaglia, F. (1993) Promiscuous and allele-specific anchors in HLA-DR-binding peptides. *Cell* **74:** 197–203.

Haneji, N., Nakamura, T., Takio, K., Yanagi, K., Higashiyama, H., Saito, I., Noji, S., Sugino, H. and Hayashi, Y. (1997) Identification of α-fodrin as a candidate autoantigen in primary Sjögren's syndrome. *Science* **276:** 604–607.

Hanson, M. S., Cetkovic-Cvrlje, M., Ramiya, V. K., Atkinson, M. A., Maclaren, N. K., Singh, B., Elliott, J. F., Serreze, D. V. and Leiter, E. H. (1996) Quantitative thresholds of MHC class II I-E expressed on hemopoietically derived antigen-presenting cells in transgenic NOD/Lt mice determine level of diabetes resistance and indicate mechanism of protection. *J. Immunol.* **157:** 1279–1287.

Harada, M., Kishimoto, Y. and Makino, S. (1990) Prevention of overt diabetes and insulitis in NOD mice by a single BCG vaccination. *Diabetes Res. Clin. Pract.* **8:** 85–89.

Harding, C. V. and Geuze, H. J. (1992) Class II MHC molecules are present in macrophage lysosomes and phagolysosomes that function in the phagocytic processing of Listeria monocytogenes for presentation to T cells. *J. Cell Biol.* **119:** 531–542.

Harding, C. V. and Geuze, H. J. (1993) Immunogenic peptides bind to class II MHC molecules in an early lysosomal compartment. *J. Immunol.* **151:** 3988–3998.

Harding, C. V. and Unanue, E. R. (1990) Quantitation of antigen-presenting cell MHC class II/peptide complexes necessary for T cell stimulation. *Nature* **346:** 574–576.

Harding, C. V., Roof, R. W. and Unanue, E. R. (1989) Turnover of Ia-peptide complexes is facilitated in viable antigen-presenting cells: Biosynthetic turnover of Ia *versus* peptide exchange. *Proc. Natl Acad. Sci. USA* **86:** 4230–4234.

Harding, C. V., Unanue, E. R., Slot, J. W., Schwartz, A. L. and Geuze, H. J. (1990) Functional and ultrastructural evidence for intracellular formation of major histocompatibility complex class II-peptide complexes during antigen processing. *Proc. Natl Acad. Sci. USA* **87:** 5553–5557.

Harding, C. V., Roof, R. W., Allen, P. M. and Unanue, E. R. (1991) Effects of pH and polysaccharides on peptide binding to class II major histocompatibility complex molecules. *Proc. Natl Acad. Sci. USA* **88:** 2740–2744.

Harlow, E. and Lane, D. (1988) Antibodies: A laboratory manual., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Harris, P. E., Maffei, A., Colovai, A. I., Kinne, J., Tugulea, S. and Suciu-Foca, N. (1996) Predominant HLA-class II bound self-peptides of a hematopoietic progenitor cell line are derived from intracellular proteins. *Blood* **87:** 5104–5112.

Harrison, L. C., Honeyman, M. C., Trembleau, S., Gregori, S., Gallazzi, F., Augstein, P., Brusic, V., Hammer, J. and Adorini, L. (1997) A peptide-binding motif for I-A^{g7}, the class II major histocompatibility complex (MHC) molecule of NOD and Biozzi AB/H mice. *J. Exp. Med.* **185:** 1013–1021.

Hattori, M., Buse, J. B., Jackson, R. A., Glimcher, L., Dorf, M. E., Minami, M., Makino, S., Kuzuya, H., Imura, H., Strauss, W. M., *et al.* (1986) The NOD mouse: recessive diabetogenic gene in the major histocompatibility complex. *Science* **231:** 733–735.

Hausmann, D. H., Yu, B., Hausmann, S. and Wucherpfennig, K. W. (1999) pH-dependent peptide binding properties of the type I diabetes-associated I-A^{g7} molecule: rapid release of CLIP at an endosomal pH. *J. Exp. Med.* **189:** 1723–1734.

Helmke, K., Otten, A., Willems, W. R., Brockhaus, R., Mueller-Eckhardt, G., Stief, T., Bertrams, J., Wolf, H. and Federlin, K. (1986) Islet cell antibodies and the development of diabetes mellitus in relation to mumps infection and mumps vaccination. *Diabetologia* **29:** 30–33.

Herman, A., Croteau, G., Sekaly, R. P., Kappler, J. and Marrack, P. (1990) HLA-DR alleles differ in their ability to present staphylococcal enterotoxins to T cells. *J. Exp. Med.* **172:** 709–717.

Hermel, E., Yuan, J. and Monaco, J. J. (1995) Characterisation of polymorphism within the H2-M MHC class II loci. *Immunogenetics* **42:** 136–142.

Herrmann, T., Accolla, R. S. and MacDonald, H. R. (1989) Different staphylococcal enterotoxins bind preferentially to distinct major histocompatibility complex class II isotypes. *Eur. J. Immunol.* **19:** 2171–2174.

Hess, A., Thoburn, C., Bright, E. and Horwitz, L. (1997a) Specificity of effector mechanisms in syngeneic graft-*versus*-host disease: recognition of the MHC class II invariant chain peptide (CLIP). *Transplant. Proc.* **29:** 725–727.

Hess, A. D., Bright, E. C., Thoburn, C., Vogelsang, G. B., Jones, R. J. and Kennedy, M. J. (1997b) Specificity of effector T lymphocytes in autologous graft-*versus*-host disease: role of the major histocompatibility complex class II invariant chain peptide. *Blood* **89:** 2203–2209.

Hess, A. D., Thoburn, C. and Horwitz, L. (1998) Promiscuous recognition of major histocompatibility complex class II determinants in cyclosporine-induced syngeneic graft-*versus*-host disease: specificity of cytolytic effector T cells. *Transplantation* **65:** 785–792.

Hitzel, C. and Koch, N. (1996) The invariant chain derived fragment CLIP is an efficient *in vitro* inhibitor of peptide binding to MHC class II molecules. *Mol. Immunol.* **33:** 25–31.

Hitzel, C., Grüneberg, U., van Ham, M., Trowsdale, J. and Koch, N. (1999) Sodium dodecyl sulfate-resistant HLA-DR "superdimer" bands are in some cases class II heterodimers bound to antibody. *J. Immunol.* **162:** 4671–4676.

Hogquist, K. A., Jameson, S. C., Heath, W. R., Howard, J. L., Bevan, M. J. and Carbone, F. R. (1994) T cell receptor antagonist peptides induce positive selection. *Cell* **76:** 17–27.

Hoover, M. L. and Marta, R. T. (1997) Molecular modelling of HLA-DQ suggests a mechanism of resistance in type 1 diabetes. *Scand. J. Immunol.* **45:** 193–202.

Horwitz, M. S., Bradley, L. M., Harbertson, J., Krahl, T., Lee, J. and Sarvetnick, N. (1998) Diabetes induced by Coxsackie virus: initiation by bystander damage and not molecular mimicry. *Nat. Med.* **4:** 781–785.

Houghten, R. A. (1985) General method for the rapid solid-phase synthesis of large numbers of peptides: specificity of antigen-antibody interaction at the level of individual amino acids. *Proc. Natl Acad. Sci. USA* **82:** 5131–5135.

Houghten, R. A., Bray, M. K., Degraw, S. T. and Kirby, C. J. (1986) Simplified procedure for carrying out simultaneous multiple hydrogen fluoride cleavages of protected peptide resins. *Int. J. Pept. Protein Res.* **27:** 673–678.

Houghton, G., Arnold, L. W., Bishop, G. A. and Mercolino, T. J. (1986) The CH series of murine B cell lymphomas: Neoplastic analogues of Ly-1$^+$ normal B cells. *Immunol. Rev.* **93:** 35–51.

Hsu, B. L., Donermeyer, D. L. and Allen, P. M. (1996) TCR recognition of the Hb(64–76)/I-E^k determinant: single conservative amino acid changes in the complementarity-determining region 3 dramatically alter antigen fine specificity. *J. Immunol.* **157:** 2291–2298.

Humphreys-Beher, M. G., Brinkley, L., Purushotham, K. R., Wang, P. L., Nakagawa, Y., Dusek, D., Kerr, M., Chegini, N. and Chan, E. K. (1993) Characterisation of antinuclear autoantibodies present in the serum from nonobese diabetic (NOD) mice. *Clin. Immunol. Immunopathol.* **68:** 350–356.

Humphreys-Beher, M. G., Hu, Y., Nakagawa, Y., Wang, P. L. and Purushotham, K. R. (1994) Utilisation of the non-obese diabetic (NOD) mouse as an animal model for the study of secondary Sjögren's syndrome. *Adv. Exp. Med. Biol.* **350:** 631–636.

Hunt, D. F., Michel, H., Dickinson, T. A., Shabanowitz, J., Cox, A. L., Sakaguchi, K., Appella, E., Grey, H. M. and Sette, A. (1992) Peptides presented to the immune system by the murine class II major histocompatibility complex molecule I-A^d. *Science* **256:** 1817–1820.

Hurley, C. K. and Steiner, N. (1995) Differences in peptide binding of DR11 and DR13 microvariants demonstrate the power of minor variation in generating DR functional diversity. *Hum. Immunol.* **43:** 101–112.

Hurtenbach, U., Lier, E., Adorini, L. and Nagy, Z. A. (1993) Prevention of autoimmune diabetes in non-obese diabetic mice by treatment with a class II major histocompatibility complex-blocking peptide. *J. Exp. Med.* **177:** 1499–1504.

Hutchings, P. R. and Cooke, A. (1990) The transfer of autoimmune diabetes in NOD mice can be inhibited or accelerated by distinct cell populations present in normal splenocytes taken from young males. *J. Autoimmun.* **3:** 175–185.

Hyoty, H., Parkkonen, P., Rode, M., Bakke, O. and Leinikki, P. (1993) Common peptide epitope in mumps virus nucleocapsid protein and MHC class II-associated invariant chain. *Scand. J. Immunol.* **37:** 550–558.

Ignatowicz, L., Kappler, J. and Marrack, P. (1996) The repertoire of T cells shaped by a single MHC/peptide ligand. *Cell* **84:** 521–529.

Ikegami, H., Makino, S., Harada, M., Eisenbarth, G. S. and Hattori, M. (1988) The cataract Shionogi mouse, a sister strain of the non-obese diabetic mouse: similar class II but different class I gene products. *Diabetologia* **31:** 254–258.

Ikegami, H., Tahara, Y., Cha, T., Yamato, E., Ogihara, T., Noma, Y. and Shima, K. (1990a) Aspartic acid at position 57 of the HLA-DQ β-chain is not protective against insulin-dependent diabetes mellitus in Japanese people. *J. Autoimmun.* **3:** 167–174.

Ikegami, H., Eisenbarth, G. S. and Hattori, M. (1990b) Major histocompatibility complex-linked diabetogenic gene of the nonobese diabetic mouse. Analysis of genomic DNA amplified by the polymerase chain reaction. *J. Clin. Invest.* **85:** 18–24.

Jacob, C. O., Aiso, S., Michie, S. A., McDevitt, H. O. and Acha-Orbea, H. (1990) Prevention of diabetes in nonobese diabetic mice by tumor necrosis factor (TNF): similarities between TNF-α and interleukin 1. *Proc. Natl Acad. Sci. USA* **87:** 968–972.

Jacobson, S., Sekaly, R. P., Jacobson, C. L., McFarland, H. F. and Long, E. O. (1989) HLA class II-restricted presentation of cytoplasmic measles virus antigens to cytotoxic T cells. *J. Virol.* **63:** 1756–1762.

Janeway, C. A. (1999) A role for self-peptides in positive selection. *Curr. Biol.* **9:** R342–R345.

Jansen, A., Homo-Delarche, F., Hooijkaas, H., Leenen, P. J., Dardenne, M. and Drexhage, H. A. (1994) Immunohistochemical characterisation of monocytes-macrophages and dendritic cells involved in the initiation of the insulitis and b-cell destruction in NOD mice. *Diabetes* **43:** 667–675.

Jaramillo, A., Gill, B. M. and Delovitch, T. L. (1994) Insulin dependent diabetes mellitus in the non-obese diabetic mouse: a disease mediated by T cell anergy? *Life Sci.* **55:** 1163–1177.

Jaraquemada, D., Marti, M. and Long, E. O. (1990) An endogenous processing pathway in vaccinia virus-infected cells for presentation of cytoplasmic antigens to class II-restricted T cells. *J. Exp. Med.* **172:** 947–954.

Jardetzky, T. S., Brown, J. H., Gorga, J. C., Stern, L. J., Urban, R. G., Chi, Y., Stauffacher, C., Strominger, J. L. and Wiley, D. C. (1994) Three-dimensional structure of a human class II histocompatibility molecule complexed with superantigen. *Nature* **368:** 711–718.

Jarpe, A. J., Hickman, M. R., Anderson, J. T., Winter, W. E. and Peck, A. B. (1990) Flow cytometric enumeration of mononuclear cell populations infiltrating the islets of Langerhans in prediabetic NOD mice: development of a model of autoimmune insulitis for type I diabetes. *Reg. Immunol.* **3:** 305–317.

Jasanoff, A., Park, S.-J. and Wiley, D. C. (1995) Direct observation of disordered regions in the major histocompatibility complex class II-associated invariant chain. *Proc. Natl Acad. Sci. USA* **92:** 9900–9904.

Jasanoff, A., Wagner, G. and Wiley, D. C. (1998) Structure of a trimeric domain of the MHC class II-associated chaperonin and targeting protein Ii. *EMBO J.* **17:** 6812–6818.

Jasanoff, A., Song, S., Dinner, A. R., Wagner, G. and Wiley, D. C. (1999) One of two unstructured domains of Ii becomes ordered in complexes with MHC class II molecules. *Immunity* **10:** 761–768.

Jensen, P. E. (1989) Stable association of processed antigen with antigen-presenting cell membranes. *J. Immunol.* **143:** 420–455.

Jensen, P. E. (1990) Regulation of antigen presentation by acidic pH. *J. Exp. Med.* **171:** 1779–1784.

Jensen, P. E. (1991) Enhanced binding of peptide antigen to purified class II major histocompatibility glycoproteins at acidic pH. *J. Exp. Med.* **174:** 1111–1120.

Jensen, P. E. (1992) Long-lived complexes between peptide and class II major histocompatibility complex are formed at low pH with no requirement for pH neutralisation. *J. Exp. Med.* **176:** 793–798.

Jiang, Z., Xu, M., Savas, L., LeClair, P. and Banner, B. F. (1999) Invariant chain expression in colon neoplasms. *Virchows Arch.* **435:** 32–36.

Johansen, B. H., Vartdal, F., Eriksen, J. A., Thorsby, E. and Sollid, L. M. (1996) Identification of a putative motif for binding of peptides to HLA-DQ2. *Int. Immunol.* **8:** 177–182.

Johnson, W. C., Jr. (1990) Protein secondary structure and circular dichroism: A practical guide. *Proteins: Structure, function and genetics* **7:** 205–214.

Joner, G. and Søvik, O. (1989) Increasing incidence of diabetes mellitus in Norwegian children 0-14 years of age 1973–1982. *Diabetologia* **32:** 79–83.

Jones, P. P., Murphy, D. B., Hewgill, D. and McDevitt, H. O. (1978a) Detection of a common polypeptide chain in *I-A* and *I-E* sub-region immunoprecipitates. *Immunochemistry* **16:** 51–60.

Jones, P. P., Murphy, D. B. and McDevitt, H. O. (1978b) Two-gene control of the expression of a murine Ia antigen. *J. Exp. Med.* **148:** 925–939.

Jones, P. P., Murphy, D. B. and McDevitt, H. O. (1981) Variable synthesis and expression of Ea and Ae (Eb) Ia polypeptide chains in mice of different H-2 haplotypes. *Immunogenetics* **12:** 321–337.

Jones, R. E., Bourdette, D. N., Offner, H. and Vandenbark, A. A. (1990) Myelin basic protein-specific T cells induce demyelinating experimental autoimmune encephalomyelitis in Buffalo rats. *J. Neuroimmunol.* **30:** 61–69.

Julier, C., Hyer, R. N., Davies, J., Merlin, F., Soularue, P., Briant, L., Cathelineau, G., Deschamps, I., Rotter, J. I., Froguel, P., *et al.* (1991) Insulin-IGF2 region on chromosome 11p encodes a gene implicated in HLA- DR4-dependent diabetes susceptibility. *Nature* **354:** 155–159.

Kabat, E. A. (1991) Sequences of proteins of immunological interest, 5th edn. National Institutes Of Health, Bethesda.

Kadlubowski, M., Hughes, R. A. and Gregson, N. A. (1980) Experimental allergic neuritis in the Lewis rat: characterisation of the activity of peripheral myelin and its major basic protein, P2. *Brain Res.* **184:** 439–454.

Kageyama, T., Yonezawa, S., Ichinose, M., Miki, K. and Moriyama, A. (1996) Potential sites for processing of the human invariant chain by cathepsins D and E. *Biochem. Biophys. Res. Commun.* **223:** 549–553.

Kagi, D., Odermatt, B., Ohashi, P. S., Zinkernagel, R. M. and Hengartner, H. (1996) Development of insulitis without diabetes in transgenic mice lacking perforin-dependent cytotoxicity. *J. Exp. Med.* **183:** 2143–2152.

Kanagawa, O., Shimizu, J. and Unanue, E. R. (1997) The role of I-A^{g7} β-chain in peptide binding and antigen recognition by T cells. *Int. Immunol.* **9:** 1523–1526.

Kanagawa, O., Martin, S. M., Vaupel, B. A., Carrasco-Marin, E. and Unanue, E. R. (1998) Autoreactivity of T cells from nonobese diabetic mice: an I-A^{g7}-dependent reaction. *Proc. Natl Acad. Sci. USA* **95:** 1721–1724.

Kanazawa, Y., Komeda, K., Sato, S., Mori, S., Akanuma, K. and Takaku, F. (1984) Non-obese-diabetic mice: immune mechanisms of pancreatic β-cell destruction. *Diabetologia* **27** (Suppl)**:** 113–115.

Kang, S., Liang, L., Parker, C. D. and Collawn, J. F. (1998) Structural requirements for major histocompatibility complex class II invariant chain endocytosis and lysosomal targeting. *J. Biol. Chem.* **273:** 20644–20652.

Kappler, J. W., Skidmore, B., White, J. and Marrack, P. (1981) Antigen-inducible, *H-2*-restricted, interleukin-2-producing T cell hybridomas. *J. Exp. Med.* **153:** 1198–1214.

Kappler, J. W., Staerz, U., White, J. and Marrack, P. C. (1988) Self-tolerance eliminates T cells specific for Mls-modified products of the major histocompatibility complex. *Nature* **332:** 35–40.

Karlsson, L. and Peterson, P. A. (1992) The α-chain gene of H-2O has an unexpected location in the major histocompatibility complex. *J. Exp. Med.* **176:** 477–483.

Karlsson, L., Surh, C. D., Sprent, J. and Peterson, P. A. (1991) A novel class II MHC molecule with unusual tissue distribution. *Nature* **351:** 485–458.

Karlsson, L., Péléraux, A., Lindstedt, R., Liljedahl, M. and Peterson, P. A. (1994) Reconstitution of an operational MHC class II compartment in nonantigen-presenting cells. *Science* **266:** 1569–1573.

Kasai, M., Hirokawa, K., Kajino, K., Ogasawara, K., Tatsumi, M., Hermel, E., Monaco, J. J. and Mizuochi, T. (1996) Difference in antigen presentation pathways between cortical and medullary thymic epithelial cells. *Eur. J. Immunol.* **26:** 2101–2107.

Katoh, M., Itoh, Y., Ogasawara, K., Kajino, K., Nishihori, H., Takahashi, A., Matsuki, N., Iwabuchi, K., Yoshida, T. O., Good, R. A., *et al.* (1996) A promiscuous T cell hybridoma restricted to various I-A molecules. *Eur. J. Immunol.* **26:** 976–980.

Katunuma, N., Kakegawa, H., Matsunaga, Y. and Saibara, T. (1994) Immunological significances of invariant chain from the aspect of its structural homology with the cystatin family. *FEBS Lett* **349:** 265-269.

Katz, J. D., Benoist, C. and Mathis, D. (1995) T helper cell subsets in insulin-dependent diabetes. *Science* **268:** 1185–1188.

Katz, J. F., Stebbins, C., Appella, E. and Sant, A. J. (1996) Invariant chain and DM edit self-peptide presentation by major histocompatibility complex (MHC) class II molecules. *J. Exp. Med.* **184:** 1747–1753.

Kaufman, J. F. and Strominger, J. L. (1982) HLA-DR light chain has a polymorphic N-terminal region and a conserved immunoglobulin-like C-terminal region. *Nature* **297:** 694–697.

Kaufman, J. F., Auffray, C., Korman, A. J., Shackelford, D. A. and Strominger, J. (1984) The class II molecules of the human and murine major histocompatibility complex. *Cell* **36:** 1–13.

Kaufman, D. L., Erlander, M. G., Clare-Salzler, M., Atkinson, M. A., Maclaren, N. K. and Tobin, A. J. (1992) Autoimmunity to two forms of glutamate decarboxylase in insulin- dependent diabetes mellitus. *J. Clin. Invest.* **89:** 283–292.

Kaufman, D. L., Clare-Salzler, M., Tian, J., Forsthuber, T., Ting, G. S., Robinson, P., Atkinson, M. A., Sercarz, E. E., Tobin, A. J. and Lehmann, P. V. (1993) Spontaneous loss of T-cell tolerance to glutamic acid decarboxylase in murine insulin-dependent diabetes. *Nature* **366:** 69–72.

Kawabe, Y. and Ochi, A. (1990) Selective anergy of Vβ8$^+$, CD4$^+$ T cells in *Staphylococcus* enterotoxin B-primed mice. *J. Exp. Med.* **172:** 1065–1070.

Kawabe, Y. and Ochi, A. (1991) Programmed cell death and extrathymic reduction of Vβ8$^+$ CD4$^+$ T cells in mice tolerant to *Staphylococcus aureus* enterotoxin B. *Nature* **349:** 245–248.

Kay, T. W., Chaplin, H. L., Parker, J. L., Stephens, L. A. and Thomas, H. E. (1997) CD4$^+$ and CD8$^+$ T lymphocytes: clarification of their pathogenic roles in diabetes in the NOD mouse. *Res. Immunol.* **148:** 320–327.

Kaye, J., Hsu, M. L., Sauron, M. E., Jameson, S. C., Gascoigne, N. R. and Hedrick, S. M. (1989) Selective development of CD4$^+$ T cells in transgenic mice expressing a class II MHC-restricted antigen receptor. *Nature* **341:** 746–749.

Keller, R. J., Eisenbarth, G. S. and Jackson, R. A. (1993) Insulin prophylaxis in individuals at high risk of type I diabetes. *Lancet* **341:** 927–928.

Kelly, A. P., Monaco, J. J., Cho, S. and Trowsdale, J. (1991) A new human HLA class II-related locus, *DM*. *Nature* **353:** 571–576.

Khanna, R., Burrows, S. R., Thomson, S. A., Moss, D. J., Cresswell, P., Poulsen, L. M. and Cooper, L. (1997) Class I processing-defective Burkitt's lymphoma cells are recognised efficiently by CD4$^+$ EBV-specific CTLs. *J. Immunol.* **158:** 3619–3625.

Kikutani, H. and Makino, S. (1992) The murine autoimmune diabetes model: NOD and related strains. *Adv. Immunol.* **51:** 285–322.

Kim, K. J., Kanellopoulos-Langevin, C., Merwin, R. M., Sachs, D. H. and Asofsky, R. (1979) Establishment and characterisation of BALB/c lymphoma cell lines with B cell properties. *J. Immunol.* **122:** 549–554.

Kim, J., Urban, R. G., Strominger, J. L. and Wiley, D. C. (1994) Toxic shock syndrome toxin-1 complexed with a class II major histocompatibility molecule HLA-DR1. *Science* **266:** 1870–1874.

Kim, D. T., Rothbard, J. B., Bloom, D. D. and Fathman, C. G. (1996) Quantitative analysis of T cell activation: role of TCR/ligand density and TCR affinity. *J. Immunol.* **156:** 2737–2742.

King, C., Davies, J., Mueller, R., Lee, M. S., Krahl, T., Yeung, B., O'Connor, E. and Sarvetnick, N. (1998) TGF-β1 alters APC preference, polarizing islet antigen responses toward a Th2 phenotype. *Immunity* **8:** 601–613.

Kinouchi, R., Kobayasi, H., Sato, K., Kimura, S. and Katagiri, M. (1994) Peptide motifs of HLA-DR4/DR53 (DRB1*0405/DRB4*0101) molecules. *Immunogenetics* **40:** 376–378.

Kirschmann, D. A., Duffin, K. L., Smith, C. E., Welply, J. K., Howard, S. C., Schwartz, B. D. and Woulfe, S. L. (1995) Naturally processed peptides from rheumatoid arthritis associated and non-associated HLA-DR alleles. *J. Immunol.* **155:** 5655–5662.

Kleijmeer, M. J., Oorschot, V. M. and Geuze, H. J. (1994) Human resident langerhans cells display a lysosomal compartment enriched in MHC class II. *J. Invest. Dermatol.* **103:** 516–523.

Kleijmeer, M. J., Ossevoort, M. A., van Veen, C. J., van Hellemond, J. J., Neefjes, J. J., Kast, W. M., Melief, C. J. and Geuze, H. J. (1995) MHC class II compartments and the kinetics of antigen presentation in activated mouse spleen dendritic cells. *J. Immunol.* **154:** 5715–5724.

Kleijmeer, M. J., Raposo, G. and Geuze, H. J. (1996) Characterisation of MHC class II compartments by immunoelectron microscopy. *Methods* **10:** 191–207.

Kleijmeer, M. J., Morkowski, S., Griffith, J. M., Rudensky, A. Y. and Geuze, H. J. (1997) Major histocompatibility complex class II compartments in human and mouse B lymphoblasts represent conventional endocytic compartments. *J. Cell Biol.* **139:** 639–649.

Klein, J. (1986) Natural history of the major histocompatibility complex, John Wiley & Sons, New York.

Klein, J. and Figueroa, F. (1981) Polymorphism of the mouse H-2 loci. *Immunol. Rev.* **60:** 23–57.

Klein, J., Juretic, A., Baxevanis, C. N. and Nagy, Z. A. (1981) The traditional and a new version of the mouse H-2 complex. *Nature* **291:** 455–460.

Koch, N. and Hämmerling, G. J. (1985) Ia-associated invariant chain is fatty acylated before addition of sialic acid. *Biochemistry* **24:** 6185–6190.

Koch, N. and Hämmerling, G. J. (1986) The HLA-D-associated invariant chain binds palmitic acid at the cysteine adjacent to the membrane segment. *J. Biol. Chem.* **261:** 3434–3440.

Koch, N., Koch, S. and Hämmerling, G. J. (1982) Ia invariant chain detected on lymphocyte surfaces by monoclonal antibody. *Nature* **299:** 644–645.

Koch, N., Lauer, W., Habicht, J. and Dobberstein, B. (1987) Primary structure of the gene for the murine Ia antigen-associated invariant chains (Ii). An alternatively spliced exon encodes a cysteine-rich domain highly homologous to a repetitive sequence of thyroglobulin. *EMBO J.* **6:** 1677–1683.

Kodama, M., Matsumoto, Y., Fujiwara, M., Masani, F., Izumi, T. and Shibata, A. (1990) A novel experimental model of giant cell myocarditis induced in rats by immunisation with cardiac myosin fraction. *Clin. Immunol. Immunopathol.* **57:** 250–262.

Koide, Y. and Yoshida, T. O. (1990) The unique nucleotide sequence of the Aβ gene in the NOD mouse is shared with its nondiabetic sister strains, the ILI and the CTS mouse. *Int. Immunol.* **2:** 189–192.

Koretz, K., Momburg, F., Otto, H. F. and Moller, P. (1987) Sequential induction of MHC antigens on autochthonous cells of ileum affected by Crohn's disease. *Am. J. Pathol.* **129:** 493–502.

Korman, A. J., Auffray, C., Schamboeck, A. and Strominger, J. L. (1982) The amino acid sequence and gene organisation of the heavy chain of the HLA-DR antigen: Homology to immunoglobulins. *Proc. Natl Acad. Sci. USA* **79:** 6013–6017.

Kotzin, B. L., Leung, D. Y., Kappler, J. and Marrack, P. (1993) Superantigens and their potential role in human disease. *Adv. Immunol.* **54:** 99–166.

Kouskoff, V., Korganow, A. S., Duchatelle, V., Degott, C., Benoist, C. and Mathis, D. (1996) Organ-specific disease provoked by systemic autoimmunity. *Cell* **87:** 811–822.

Kovacsovics-Bankowski, M., Clark, K., Benacerraf, B. and Rock, K. L. (1993) Efficient major histocompatibility complex class I presentation of exogenous antigen upon phagocytosis by macrophages. *Proc. Natl Acad. Sci. USA* **90:** 4942–4946.

Kovats, S., Grubin, C. E., Eastman, S., deRoos, P., Dongre, A., Van Kaer, L. and Rudensky, A. Y. (1998) Invariant chain-independent function of H-2M in the formation of endogenous peptide-major histocompatibility complex class II complexes in vivo. *J. Exp. Med.* **187:** 245–251.

Kristensen, N. M., Hoyne, G. F., Hayball, J. D., Hetzel, C., Bourne, T. and Lamb, J. R. (1996) Induction of T cell responses to the invariant chain derived peptide CLIP in mice immunized with the group 1 allergen of house dust mite. *Int. Immunol.* **8:** 1091–1098.

Kropshofer, H., Max, H., Halder, T., Kalbus, M., Muller, C. A. and Kalbacher, H. (1993) Self-peptides from four HLA-DR alleles share hydrophobic anchor residues near the NH_2-terminal including proline as a stop signal for trimming. *J. Immunol.* **151:** 4732–4742.

Kropshofer, H., Vogt, A. B. and Hämmerling, G. J. (1995a) Structural features of the invariant chain fragment CLIP controlling rapid release from HLA-DR molecules and inhibition of peptide binding. *Proc. Natl Acad. Sci. USA* **92:** 8313–8317.

Kropshofer, H., Vogt, A. B., Stern, L. J. and Hämmerling, G. J. (1995b) Self-release of CLIP in peptide loading of HLA-DR molecules. *Science* **270:** 1357–1359.

Kropshofer, H., Vogt, A. B., Moldenhauer, G., Hammer, J., Blum, J. S. and Hämmerling, G. J. (1996) Editing of the HLA-DR–peptide repertoire by HLA-DM. *EMBO J.* **15:** 6144–6154.

Kropshofer, H., Arndt, S. O., Moldenhauer, G., Hämmerling, G. J. and Vogt, A. B. (1997) HLA-DM acts as a molecular chaperone and rescues empty HLA-DR molecules at lysosomal pH. *Immunity* **6:** 293–302.

Kropshofer, H., Vogt, A. B., Thery, C., Armandola, E. A., Li, B. C., Moldenhauer, G., Amigorena, S. and Hämmerling, G. J. (1998) A role for HLA-DO as a co-chaperone of HLA-DM in peptide loading of MHC class II molecules. *EMBO J.* **17:** 2971–2981.

Krug, J., Williams, A. J., Beales, P. E., Doniach, I., Gale, E. A. and Pozzilli, P. (1991) Parathyroiditis in the non-obese diabetic mouse — a new finding. *J. Endocrinol.* **131:** 193–196.

Ktistakis, N. T., Thomas, D. and Roth, M. G. (1990) Characteristics of the tyrosine recognition signal for internalisation of transmembrane surface glycoproteins. *J. Cell Biol.* **111:** 1393–1407.

Kudo, J., Chao, L. Y., Narni, F. and Saunders, G. F. (1985) Structure of the human gene encoding the invariant gamma-chain of class II histocompatibility antigens. *Nucleic Acids Res.* **13:** 8827–8841.

Kumar, V., Bhardwaj, V., Soares, L., Alexander, J., Sette, A. and Sercarz, E. (1995) Major histocompatibility complex binding affinity of an antigenic determinant is crucial for the differential secretion of interleukin 4/5 or interferon-γ by T cells. *Proc. Natl Acad. Sci. USA* **92:** 9510–9014.

Kurrer, M. O., Pakala, S. V., Hanson, H. L. and Katz, J. D. (1997) B cell apoptosis in T cell-mediated autoimmune diabetes. *Proc. Natl Acad. Sci. USA* **94:** 213–218.

Kuwana, T., Peterson, P. A. and Karlsson, L. (1998) Exit of major histocompatibility complex class II-invariant chain p35 complexes from the endoplasmic reticulum is modulated by phosphorylation. *Proc. Natl Acad. Sci. USA* **95:** 1056–1061.

Kwok, W. K., Domeier, M. E., Raymond, F. C., Byers, P. and Nepom, G. T. (1996a) Allele-specific motifs characterise HLA-DQ interactions with a diabetes-associated peptide derived from glutamic acid decarboxylase. *J. Immunol.* **156:** 2171–2177.

Kwok, W. W., Domeier, M. E., Johnson, M. L., Nepom, G. T. and Koelle, D. M. (1996b) HLA-DQB1 codon 57 is critical for peptide binding and recognition. *J. Exp. Med.* **183:** 1253–1258.

L'Abbe, D., Tremblay, L., Filion, M., Busque, L., Goldman, M., Decary, F. and Chartrand, P. (1992) Alloimmunization to platelet antigen HPA-1a (PIA1) is strongly associated with both HLA-DRB3*0101 and HLA-DQB1*0201. *Hum. Immunol.* **34:** 107–114.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **277:** 680–685.

Lafferty, K. J. (1997) Immunobiology of autoimmune diabetes. *Res. Immunol.* **148:** 313–319.

Lafuse, W. P., Brown, D., Castle, L. and Zwilling, B. S. (1995) IFN-gamma increases cathepsin H mRNA levels in mouse macrophages. *J. Leukoc. Biol.* **57:** 663–669.

Lah, T. T., Hawley, M., Rock, K. L. and Goldberg, A. L. (1995) Gamma-interferon causes a selective induction of the lysosomal proteases, cathepsins B and L, in macrophages. *FEBS Lett.* **363:** 85–89.

Lamb, C. A. and Cresswell, P. (1992) Assembly and transport properties of invariant chain trimers and HLA-DR–invariant chain complexes. *J. Immunol.* **148:** 3478–3482.

Lamont, A. G., Powell, M. F., Colon, S. M., Miles, C., Grey, H. M. and Sette, A. (1990) The use of peptide analogues with improved stability and MHC binding capacity to inhibit antigen presentation *in vitro* and *in vivo*. *J. Immunol.* **144:** 2493–2498.

Lanzavecchia, A. (1987) Antigen uptake and accumulation in antigen-specific B cells. *Immunol. Rev.* **99:** 39–51.

Lanzavecchia, A. (1990) Receptor-mediated antigen uptake and its effect on antigen presentation to class II-restricted T lymphocytes. *Annu. Rev. Immunol.* **8:** 773-793.

Lanzavecchia, A. (1997) Understanding the mechanisms of sustained signaling and T cell activation. *J. Exp. Med.* **185:** 1717–1719.

Lanzavecchia, A., Reid, P. A. and Watts, C. (1992) Irreversible association of peptides with class II MHC molecules in living cells. *Nature* **357:** 249–252.

Lanzavecchia, A., Lezzi, G. and Viola, A. (1999) From TCR engagement to T cell activation: a kinetic view of T cell behavior. *Cell* **96:** 1–4.

Larhammar, D., Gustafsson, K., Claesson, L., Bill, P., Wiman, K., Schenning, L., Sundelin, J., Widmark, E., Peterson, P. A. and Rask, L. (1982) α-chain of HLA-DR transplantation antigens is a member of the same protein superfamily as the immunoglobulins. *Cell* **30:** 153–161.

Larhammar, D., Hämmerling, U., Rask, L. and Peterson, P. A. (1985) Sequence of gene and cDNA encoding murine major histocompatibility complex class II gene *Aβ2*. *J. Biol. Chem.* **260:** 14111–14119.

Lavoie, P. M., Thibodeau, J., Erard, F. and Sekaly, R. P. (1999) Understanding the mechanism of action of bacterial superantigens from a decade of research. *Immunol. Rev.* **168:** 257–269.

Lechler, R., Aichinger, G. and Lightstone, L. (1996) The endogenous pathway of MHC class II antigen presentation. *Immunol. Rev.* 51–79.

Lee, C. and McConnell, H. M. (1995) A general model of invariant chain association with class II major histocompatibility complex proteins. *Proc. Natl Acad. Sci. USA* **92:** 8269–8273.

Lee, J. M. and Watts, T. H. (1990) On the dissociation and reassociation of MHC class II-foreign peptide complexes. Evidence that brief transit through an acidic compartment is not sufficient for binding site regeneration. *J. Immunol.* **144:** 1829–1834.

Lee, J. S., Trowsdale, J., Travers, P. J., Carey, J., Grosveld, F., Jenkins, J. and Bodmer, W. F. (1982) Sequence of an HLA-DR α-chain cDNA clone and intron-exon organisation of the corresponding gene. *Nature* **299:** 750–752.

Lee, J. M., Kay, C. M. and Watts, T. H. (1992) Conformational changes in mouse MHC class II molecules at acidic pH. *Int. Immunol.* **4:** 889–897.

Lee, C., Liang, M. N., Tate, K. M., Rabinowitz, J. D., Beeson, C., Jones, P. P. and McConnell, H. M. (1998) Evidence that the autoimmune antigen myelin basic protein (MBP) Ac1–9 binds towards one end of the major histocompatibility complex (MHC) cleft. *J. Exp. Med.* **187:** 1505–1516.

Lee, D. S., Ahn, C., Ernst, B., Sprent, J. and Surh, C. D. (1999) Thymic selection by a single MHC/peptide ligand: autoreactive T cells are low-affinity cells. *Immunity* **10:** 83–92.

Legge, K. L., Min, B., Pack, C., Caprio, J. and Zaghouani, H. (1999) Differential presentation of an altered peptide within fetal central and peripheral organs supports an avidity model for thymic T cell development and implies a peripheral readjustment for activation. *J. Immunol.* **162:** 5738–5746.

Leighton, J., Sette, A., Sidney, J., Appella, E., Ehrhardt, C., Fuchs, S. and Adorini, L. (1991) Comparison of structural requirements for interaction of the same peptide with $I\text{-}E^k$ and $I\text{-}E^d$ molecules in the activation of MHC class II-restricted T cells. *J. Immunol.* **147:** 198–204.

Leiter, E. H., Serreze, D. V. and Prochazka, M. (1990) The genetics and epidemiology of diabetes in NOD mice. *Immunol. Today* **11:** 147–149.

Lenarcic, B. and Bevec, T. (1998) Thyropins — new structurally related proteinase inhibitors. *Biol. Chem.* **379:** 105–111.

Lenschow, D. J., Ho, S. C., Sattar, H., Rhee, L., Gray, G., Nabavi, N., Herold, K. C. and Bluestone, J. A. (1995) Differential effects of anti-B7-1 and anti-B7-2 monoclonal antibody treatment on the development of diabetes in the nonobese diabetic mouse. *J. Exp. Med.* **181:** 1145–1155.

Leung, D. Y., Harbeck, R., Bina, P., Reiser, R. F., Yang, E., Norris, D. A., Hanifin, J. M. and Sampson, H. A. (1993) Presence of IgE antibodies to staphylococcal exotoxins on the skin of patients with atopic dermatitis. Evidence for a new group of allergens. *J. Clin. Invest.* **92:** 1374–1380.

Leung, D. Y., Travers, J. B., Giorno, R., Norris, D. A., Skinner, R., Aelion, J., Kazemi, L. V., Kim, M. H., Trumble, A. E., Kotb, M., *et al.* (1995) Evidence for a streptococcal superantigen-driven process in acute guttate psoriasis. *J. Clin. Invest.* **96:** 2106–2112.

Li, X., Golden, J. and Faustman, D. L. (1993) Faulty major histocompatibility complex class II I-E expression is associated with autoimmunity in diverse strains of mice. Autoantibodies, insulitis, and sialadenitis. *Diabetes* **42:** 1166–1172.

Li, H., Llera, A., Tsuchiya, D., Leder, L., Ysern, X., Schlievert, P. M., Karjalainen, K. and Mariuzza, R. A. (1998) Three-dimensional structure of the complex between a T cell receptor β-chain and the superantigen staphylococcal enterotoxin B. *Immunity* **9:** 807–816.

Li, H., Llera, A., Malchiodi, E. L. and Mariuzza, R. A. (1999) The structural basis of T cell activation by superantigens. *Annu. Rev. Immunol.* **17:** 435–466.

Liang, M. N., Beeson, C., Mason, K. and McConnell, H. M. (1995) Kinetics of the reactions between the invariant chain (85–99) peptide and proteins of the murine class II MHC. *Int. Immunol.* **7:** 1397–1404.

Liang, M. N., Lee, C., Xia, Y. and McConnell, H. M. (1996) Molecular modeling and design of invariant chain peptides with altered dissociation kinetics from class II MHC. *Biochemistry* **35:** 14734–14742.

Liblau, R. S., Singer, S. M. and McDevitt, H. O. (1995) Th1 and Th2 $CD4^+$ T cells in the pathogenesis of organ-specific autoimmune diseases. *Immunol. Today* **16:** 34–38.

Lightstone, L., Hargreaves, R., Bobek, G., Peterson, M., Aichinger, G., Lombardi, G. and Lechler, R. (1997) In the absence of the invariant chain, HLA-DR molecules display a distinct array of peptides which is influenced by the presence or absence of HLA-DM. *Proc. Natl Acad. Sci. USA* **94:** 5772–5777.

Liljedahl, M., Kuwana, T., Fung-Leung, W. P., Jackson, M. R., Peterson, P. A. and Karlsson, L. (1996) HLA-DO is a lysosomal resident which requires association with HLA-DM for efficient intracellular transport. *EMBO J.* **15:** 4817–4824.

Liljedahl, M., Winqvist, O., Surh, C. D., Wong, P., Ngo, K., Teyton, L., Peterson, P. A., Brunmark, A., Rudensky, A. Y., Fung-Leung, W. P., *et al.* (1998) Altered antigen presentation in mice lacking H2-O. *Immunity* **8:** 233–243.

Lindner, R. and Unanue, E. R. (1996) Distinct antigen MHC class II complexes generated by separate processing pathways. *EMBO J.* **15:** 6910–6920.

Lindstedt, R., Liljedahl, M., Peleraux, A., Peterson, P. A. and Karlsson, L. (1995) The MHC class II molecule H2-M is targeted to an endosomal compartment by a tyrosine-based targeting motif. *Immunity* **3:** 561–572.

Lindstrom, J. (1979) Autoimmune response to acetylcholine receptors in myasthenia gravis and its animal model. *Adv. Immunol.* **27:** 1–50.

Liou, W., Geuze, H. J., Geelen, M. J. and Slot, J. W. (1997) The autophagic and endocytic pathways converge at the nascent autophagic vacuoles. *J. Cell Biol.* **136:** 61–70.

Lipp, J. and Dobberstein, B. (1986) Signal recognition particle-dependent membrane insertion of mouse invariant chain: a membrane-spanning protein with a cytoplasmically exposed amino terminus. *J. Cell Biol.* **102:** 2169–2175.

Liu, G. Y. and Wraith, D. C. (1995) Affinity for class II MHC determines the extent to which soluble peptides tolerize autoreactive T cells in naive and primed adult mice-implications for autoimmunity. *Int. Immunol.* **7:** 1255–1263.

Liu, G. Y., Baker, D., Fairchild, S., Figueroa, F., Quartey-Papafio, R., Tone, M., Healey, D., Cooke, A. and Wraith, D. C. (1993) Complete characterisation of the expressed immune response genes in Biozzi AB/H mice: structural and functional identity between AB/H and NOD *A* region molecules. *Immunogenetics* **37:** 296–300.

Liu, G. Y., Fairchild, P. J., Smith, R. M., Prowle, J. R., Kioussis, D. and Wraith, D. C. (1995) Low avidity recognition of self-antigen by T cells permits escape from central tolerance. *Immunity* **3:** 407–415.

Liu, S. H., Marks, M. S. and Brodsky, F. M. (1998) A dominant-negative clathrin mutant differentially affects trafficking of molecules with distinct sorting motifs in the class II major histocompatibility complex (MHC) pathway. *J. Cell Biol.* **140:** 1023–1037.

Ljunggren, H. G., Stam, N. J., Ohlen, C., Neefjes, J. J., Hoglund, P., Heemels, M. T., Bastin, J., Schumacher, T. N., Townsend, A., Karre, K., *et al.* (1990) Empty MHC class I molecules come out in the cold. *Nature* **346:** 476–480.

Lo, D. and Sprent, J. (1986) Identity of cells that imprint H-2-restricted T-cell specificity in the thymus. *Nature* **319:** 672–675.

Long, E. O., LaVaute, T., Pinet, V. and Jaraquemada, D. (1994) Invariant chain prevents the HLA-DR-restricted presentation of a cytosolic peptide. *J. Immunol.* **153:** 1487–1494.

Loss, G. E., Jr. and Sant, A. J. (1993) Invariant chain retains MHC class II molecules in the endocytic pathway. *J. Immunol.* **150:** 3187–3197.

Loss, G. E., Jr., Elias, C. G., Fields, P. E., Ribaudo, R. K., McKisic, M. and Sant, A. J. (1993) Major histocompatibility complex class II-restricted presentation of an internally-synthesized antigen displays cell-type variability and segregates from the exogenous class II and endogenous class I presentation pathways. *J. Exp. Med.* **178:** 73–85.

Lotteau, V., Teyton, L., Peleraux, A., Nilsson, T., Karlsson, L., Schmid, S. L., Quaranta, V. and Peterson, P. A. (1990) Intracellular transport of class II MHC molecules directed by invariant chain. *Nature* **348:** 600–605.

Lu, S., Reyes, V. E., Lew, R. A., Anderson, J., Mole, J., Humphreys, R. E. and Ciardelli, T. (1990) Role of recurrent hydrophobic residues in catalysis of helix formation by T cell-presented peptides in the presence of lipid vesicles. *J. Immunol.* **145:** 899–904.

Lu, Y., Ussery, G. D., Jacim, M., Tschickardt, M., Boss, J. M. and Blanck, G. (1994) Retinoblastoma protein regulation of surface CD74 (invariant chain) expression in breast carcinoma cells. *Mol. Immunol.* **31:** 1365–1368.

Lucassen, A. M., Julier, C., Beressi, J. P., Boitard, C., Froguel, P., Lathrop, M. and Bell, J. I. (1993) Susceptibility to insulin dependent diabetes mellitus maps to a 4.1 kb segment of DNA spanning the insulin gene and associated VNTR. *Nat. Genet.* **4:** 305–310.

Lühder, F., Katz, J., Benoist, C. and Mathis, D. (1998) Major histocompatibility complex class II molecules can protect from diabetes by positively selecting T cells with additional specificities. *J. Exp. Med.* **187:** 379–387.

Lund, T., O'Reilly, L., Hutchings, P., Kanagawa, O., Simpson, E., Gravely, R., Chandler, P., Dyson, J., Picard, J. K., Edwards, A., *et al.* (1990a) Prevention of insulin-dependent diabetes mellitus in non-obese diabetic mice by transgenes encoding modified I-A β-chain or normal I-E α-chain. *Nature* **345:** 727–729.

Lund, T., Simpson, E. and Cooke, A. (1990b) Restriction fragment length polymorphisms in the major histocompatibility complex of the non-obese diabetic mouse. *J. Autoimmun.* **3:** 289–298.

Lundin, K. E., Gaudernack, G., Qvigstad, E., Sollid, L. M. and Thorsby, E. (1988) T lymphocyte clones recognizing an HLA-DQw3.2-associated epitope involving residue 57 on the DQ β-chain. *Hum. Immunol.* **22:** 235–246.

Luppi, P. and Trucco, M. (1996) Superantigens in insulin-dependent diabetes mellitus. *Springer Semin. Immunopathol.* **17:** 333–362.

Lyons, D. S., Lieberman, S. A., Hampl, J., Boniface, J. J., Chien, Y., Berg, L. J. and Davis, M. M. (1996) A TCR binds to antagonist ligands with lower affinities and faster dissociation rates than to agonists. *Immunity* **5:** 53–61.

MacDonald, H. R., Schneider, R., Lees, R. K., Howe, R. C., Acha-Orbea, H., Festenstein, H., Zinkernagel, R. M. and Hengartner, H. (1988) T-cell receptor Vβ use predicts reactivity and tolerance to *Mls*^{a}-encoded antigens. *Nature* **332:** 40–45.

Machamer, C. E. and Cresswell, P. (1982) Biosynthesis and glycosylation of the invariant chain associated with HLA-DR antigens. *J. Immunol.* **129:** 2564–2569.

Machamer, C. E. and Cresswell, P. (1984) Monensin prevents terminal glycosylation of the N- and O-linked oligosaccharides of the HLA-DR-associated invariant chain and inhibits its dissociation from the αβ-chain complex. *Proc. Natl Acad. Sci. USA* **81:** 1287–1291.

Machy, P., Bizozzero, J. P., Reggio, H. and Leserman, L. (1990) Endocytosis and recycling of MHC-encoded class II molecules by mouse B lymphocytes. *J. Immunol.* **145:** 1350–1355.

Madden, D. R., Gorga, J. C., Strominger, J. L. and Wiley, D. C. (1991) The structure of HLA-B27 reveals nonamer self-peptides bound in an extended conformation. *Nature* **353:** 321–325.

Madden, D. R., Gorga, J. C., Strominger, J. L. and Wiley, D. C. (1992) The three-dimensional structure of HLA-B27 at 2.1 Å resolution suggests a general mechanism for tight peptide binding to MHC. *Cell* **70:** 1035–1048.

Maeda, T., Sumida, T., Kurasawa, K., Tomioka, H., Itoh, I., Yoshida, S. and Koike, T. (1991) T-lymphocyte-receptor repertoire of infiltrating T lymphocytes into NOD mouse pancreas. *Diabetes* **40:** 1580–1585.

Makino, S., Kunimoto, K., Muraoka, Y., Mizushima, Y., Katagiri, K. and Tochino, Y. (1980) Breeding of a non-obese, diabetic strain of mice. *Jikken Dobutsu* **29:** 1–13.

Makino, S., Kunimoto, K., Muraoka, Y. and Katagiri, K. (1981) Effect of castration on the appearance of diabetes in NOD mice. *Jikken Dobutsu* **30:** 137–140.

Makino, S., Harada, M., Kishimoto, Y. and Hayashi, Y. (1986) Absence of insulitis and overt diabetes in athymic nude mice with NOD genetic background. *Jikken Dobutsu* **35:** 495–498.

Makino, S., Kishimoto, Y., Kunimoto, K., Kawaguchi, J. and Uchida, K. (1991) Localisation of the MHC-linked diabetogenic genes of the NOD mouse by using congenic strains. *Diabetes Res. Clin. Pract.* **14:** S40 (Abstr.).

Malaisse, W. J., Malaisse-Lagae, F., Sener, A. and Pipeleers, D. G. (1982) Determinants of the selective toxicity of alloxan to the pancreatic B cell. *Proc. Natl Acad. Sci. USA* **79:** 927–930.

Malcherek, G., Gnau, V., G., J., Rammensee, H.-G. and Melms, A. (1995) Supermotifs enable natural invariant chain-derived peptides to interact with many major histocompatibility complex-class II molecules. *J. Exp. Med.* **181:** 527–536.

Malcherek, G., Wirblich, C., Willcox, N., Rammensee, H.-G., Trowsdale, J. and Melms, A. (1998) MHC class II-associated invariant chain peptide replacement by T cell epitopes: engineered invariant chain as a vehicle for directed and enhanced MHC class II antigen processing and presentation. *Eur. J. Immunol.* **28:** 1524–1533.

Malnati, M. S., Marti, M., LaVaute, T., Jaraquemada, D., Biddison, W., DeMars, R. and Long, E. O. (1992) Processing pathways for presentation of cytosolic antigen to MHC class II-restricted T cells. *Nature* **357:** 702–704.

Malnati, M. S., Ceman, S., Weston, M., DeMars, R. and Long, E. O. (1993) Presentation of cytosolic antigen by HLA-DR requires a function encoded in the class II region of the MHC. *J. Immunol.* **151:** 6751–6756.

Mandrup-Poulsen, T., Helqvist, S., Wogensen, L. D., Molvig, J., Pociot, F., Johannesen, J. and Nerup, J. (1990) Cytokine and free radicals as effector molecules in the destruction of pancreatic β cells. *Curr. Top. Microbiol. Immunol.* **164:** 169–193.

Manoury, B., Hewitt, E. W., Morrice, N., Dando, P. M., Barrett, A. J. and Watts, C. (1998) An asparaginyl endopeptidase processes a microbial antigen for class II MHC presentation. *Nature* **396:** 695–699.

Mantovani, V., Corazza, G. R., Bragliani, M., Frisoni, M., Zaniboni, M. G. and Gasbarrini, G. (1993) Asp57-negative HLA DQ β-chain and DQA1*0501 allele are essential for the onset of DQw2-positive and DQw2-negative coeliac disease. *Clin. Exp. Immunol.* **91:** 153–156.

Maric, M. A., Taylor, M. D. and Blum, J. S. (1994) Endosomal aspartic proteinases are required for invariant-chain processing. *Proc. Natl Acad. Sci. USA* **91:** 2171–2175.

Marks, M. S., Blum, J. S. and Cresswell, P. (1990) Invariant chain trimers are sequestered in the rough endoplasmic reticulum in the absence of association with HLA class II antigens. *J. Cell Biol.* **111:** 839–855.

Marks, M. S., Germain, R. N. and Bonifacino, J. S. (1995a) Transient aggregation of major histocompatibility complex class II chains during assembly in normal spleen cells. *J. Biol. Chem.* **270:** 10475–10481.

Marks, M. S., Roche, P. A., van Donselaar, E., Woodruff, L., Peters, P. J. and Bonifacino, J. S. (1995b) A lysosomal targeting signal in the cytoplasmic tail of the β-chain directs HLA-DM to MHC class II compartments. *J. Cell Biol.* **131:** 351–369.

Marrack, P., Ignatowicz, L., Kappler, J. W., Boymel, J. and Freed, J. H. (1993) Comparison of peptides bound to spleen and thymus class II. *J. Exp. Med.* **178:** 2173–2183.

Marsh, E. W., Dalke, D. P. and Pierce, S. K. (1992) Biochemical evidence for the rapid assembly and disassembly of processed antigen-major histocompatibility complex class II complexes in acidic vesicles of B cells. *J. Exp. Med.* **175:** 425–436.

Marshall, K. W., Liu, A. F., Canales, J., Perahia, B., Jorgensen, B., Gantzos, R. D., Aguilar, B., Devaux, B. and Rothbard, J. B. (1994) Role of the polymorphic residues in HLA-DR molecules in allele-specific binding of peptide ligands. *J. Immunol.* **152:** 4946–4957.

Martin, W. D., Hicks, G. G., Mendiratta, S. K., Leva, H. I., Ruley, H. E. and Van Kaer, L. (1996) H2-M mutant mice are defective in the peptide loading of class II molecules, antigen presentation, and T cell repertoire selection. *Cell* **84:** 543–550.

Mason, K., Denney, D. J. and McConnell, H. M. (1995) Myelin basic protein peptide complexes with the class II MHC molecules I-A^s and I-A^k form and dissociate rapidly at neutral pH. *J. Immunol.* **154:** 5216–5227.

Mathis, D. J., Benoist, C., Williams, V. E. D., Kanter, M. and McDevitt, H. O. (1983) Several mechanisms can account for defective *Ea* gene expression in different mouse haplotypes. *Proc. Natl Acad. Sci. USA* **80:** 273–277.

Matsui, K., Boniface, J. J., Steffner, P., Reay, P. A. and Davis, M. M. (1994) Kinetics of T-cell receptor binding to peptide/I-E^k complexes: Correlation of the dissociation rate with T-cell responsiveness. *Proc. Natl Acad. Sci. USA* **91:** 12862–12866.

Mazza, G., Housset, D., Piras, C., Gregoire, C., Lin, S. Y., Fontecilla-Camps, J. C. and Malissen, B. (1998) Glimpses at the recognition of peptide/MHC complexes by T-cell antigen receptors. *Immunol. Rev.* **163:** 187–196.

McCulloch, D. K., Klaff, L. J., Kahn, S. E., Schoenfeld, S. L., Greenbaum, C. J., Mauseth, R. S., Benson, E. A., Nepom, G. T., Shewey, L. and Palmer, J. P. (1990) Nonprogression of subclinical β-cell dysfunction among first-degree relatives of IDDM patients. 5-yr follow-up of the Seattle Family Study. *Diabetes* **39:** 549–556.

McGrath, M. E. (1999) The lysosomal cysteine proteases. *Annu. Rev. Biophys. Biomol. Struct.* **28:** 181–204.

McKeever, U., Khandekar, S., Newcomb, J., Naylor, J., Gregory, P., Brauer, P., Jesson, M., Bettencourt, B., Burke, E., Alderson, A., *et al.* (1996) Immunisation with soluble BDC 2.5 T cell receptor-immunoglobulin chimeric protein:antibody specificity and protection of nonobese diabetic mice against adoptive transfer of diabetes by maternal immunization. *J. Exp. Med.* **184:** 1755–1768.

McKnight, A. J., Mason, D. W. and Barclay, A. N. (1989) Sequence of a rat MHC class II-associated invariant chain cDNA clone containing a 64 amino acid thyroglobulin-like domain. *Nucleic Acids Res.* **17:** 3983–3984.

McMaster, W. R. (1981) A monoclonal antibody which detects a polymorphic Ia antigenic determinant reacts with purified b polypeptide chain. *Immunogenetics* **13:** 347–350.

McMaster, W. R. and Williams, A. F. (1979) Identification of Ia glycoproteins in rat thymus and purification from rat spleen. *Eur. J. Immunol.* **9:** 426–433.

McMillan, M., Frelinger, J. A., Jones, P. P., Murphy, D. B., McDevitt, H. O. and Hood, L. (1981) Structure of murine Ia antigens: Two-dimensional electrophoretic analyses and high-pressure liquid chromatography tryptic peptide maps of products of the *I-A* and *I-E* subregions and of an associated invariant polypeptide. *J. Exp. Med.* **153:** 936–950.

McNicholas, J., Steinmetz, M., Hunkapiller, T., Jones, P. and Hood, L. (1982) DNA sequence of the gene encoding the Eα Ia polypeptide of the BALB/c mouse. *Science* **218:** 1229–1232.

Medema, J. P. and Borst, J. (1999) T cell signaling: a decision of life and death. *Hum. Immunol.* **60:** 403–411.

Mehringer, J. H., Harris, M. R., Kindle, C. S., McCourt, D. W. and Cullen, S. E. (1991) Characterisation of fragments of the murine Ia-associated invariant chain. *J. Immunol.* **146:** 920–927.

Mellins, E., Smith, L., Arp, B., Cotner, T., Celis, E. and Pious, D. (1990) Defective processing and presentation of exogenous antigens in mutants with normal HLA class II genes. *Nature* **343:** 71–74.

Mellins, E., Kempin, S., Smith, L., Monji, T. and Pious, D. (1991) A gene required for class II-restricted antigen presentation maps to the major histocompatibility complex. *J. Exp. Med.* **174:** 1607–1615.

Mellins, E., Cameron, P., Amaya, M., Goodman, S., Pious, D., Smith, L. and Arp, B. (1994) A mutant human histocompatibility leukocyte antigen DR molecule associated with invariant chain peptides. *J. Exp. Med.* **179:** 541–549.

Mengle-Gaw, L. and McDevitt, H. O. (1983) Isolation and characterisation of a cDNA clone of the murine I-Eβ polypeptide chain. *Proc. Natl Acad. Sci. USA* **80:** 7621–7625.

Mengle-Gaw, L. and McDevitt, H. O. (1985) Genetics and expression of mouse Ia antigens. *Annu. Rev. Immunol.* **3:** 367–396.

Metzelaar, M. J., Wijngaard, P. L., Peters, P. J., Sixma, J. J., Nieuwenhuis, H. K. and Clevers, H. C. (1991) CD63 antigen. A novel lysosomal membrane glycoprotein, cloned by a screening procedure for intracellular antigens in eukaryotic cells. *J. Biol. Chem.* **266:** 3239–3245.

Michel, C., Boitard, C. and Bach, J. F. (1989) Insulin autoantibodies in non-obese diabetic (NOD) mice. *Clin. Exp. Immunol.* **75:** 457–460.

Miller, J., Hatch, J. A., Simonis, S. and Cullen, S. E. (1988) Identification of the glycosaminoglycan-attachment site of mouse invariant-chain proteoglycan core protein by site-directed mutagenesis. *Proc. Natl Acad. Sci. USA* **85:** 1359–1363.

Mitchell, R. N., Barnes, K. A., Grupp, S. A., Sanchez, M., Misulovin, Z., Nussenzweig, M. C. and Abbas, A. K. (1995) Intracellular targeting of antigens internalised by membrane immunoglobulin in B lymphocytes. *J. Exp. Med.* **181:** 1705–1714.

Miyagawa, J., Hanafusa, T., Miyazaki, A., Yamada, K., Fujino-Kurihara, H., Nakajima, H., Kono, N., Nonaka, K., Tochino, Y. and Tarui, S. (1986) Ultrastructural and immunocytochemical aspects of lymphocytic submandibulitis in the non-obese diabetic (NOD) mouse. *Virchows Arch. B Cell. Pathol.* **51:** 215–225.

Miyagawa, J., Yamamoto, K., Hanafusa, T., Itoh, N., Nakagawa, C., Otsuka, A., Katsura, H., Yamagata, K., Miyazaki, A., Kono, N., *et al.* (1990) Preventive effect of a new immunosuppressant FK-506 on insulitis and diabetes in non-obese diabetic mice. *Diabetologia* **33:** 503–505.

Miyazaki, A., Hanafusa, T., Yamada, K., Miyagawa, J., Fujino-Kurihara, H., Nakajima, H., Nonaka, K. and Tarui, S. (1985) Predominance of T lymphocytes in pancreatic islets and spleen of pre-diabetic non-obese diabetic (NOD) mice: a longitudinal study. *Clin. Exp. Immunol.* **60:** 622–630.

Miyazaki, T., Uno, M., Uehira, M., Kikutani, H., Kishimoto, T., Kimoto, M., Nishimoto, H., Miyazaki, J. and Yamamura, K. (1990) Direct evidence for the contribution of the unique $I\text{-}A^{NOD}$ to the development of insulitis in non-obese diabetic mice. *Nature* **345:** 722–724.

Miyazaki, T., Wolf, P., Tourne, S., Waltzinger, C., Dierich, A., Barois, N., Ploegh, H., Benoist, C. and Mathis, D. (1996) Mice lacking H2-M complexes, enigmatic elements of the MHC class II peptide-loading pathway. *Cell* **84:** 531–541.

Mizuochi, T., Yee, S. T., Kasai, M., Kakiuchi, T., Muno, D. and Kominami, E. (1994) Both cathepsin B and cathepsin D are necessary for processing of ovalbumin as well as for degradation of class II MHC invariant chain. *Immunol. Lett.* **43:** 189–193.

Momburg, F., Koretz, K., Von Herbay, A. and Moller, P. (1988) Nonimmune human cells can express MHC class II antigens in the absence of invariant chain — an immunohistological study on normal and chronically inflamed small intestine. *Clin. Exp. Immunol.* **72:** 367–372.

Monji, T., McCormack, A. L., Yates, J. R. I. and Pious, D. (1994) Invariant-cognate peptide exchange restores class II dimer stability in HLA-DM mutants. *J. Immunol.* **153:** 4468–4477.

Monji, T. and Pious, D. (1997) Exogenously provided peptides fail to complex with intracellular class II molecules for presentation by antigen-presenting cells. *J. Immunol.* **158:** 3155–3164.

Morel, P. A., Livingstone, A. M. and Fathman, C. G. (1987) Correlation of T cell receptor Vβ gene family with MHC restriction. *J. Exp. Med.* **166:** 583–588.

Morel, P. A., Dorman, J. S., Todd, J. A., McDevitt, H. O. and Trucco, M. (1988) Aspartic acid at position 57 of the HLA-DQ β-chain protects against type I diabetes: a family study. *Proc. Natl Acad. Sci. USA* **85:** 8111–8115.

Mori, Y., Suko, M., Okudaira, H., Matsuba, I., Tsuruoka, A., Sasaki, A., Yokoyama, H., Tanase, T., Shida, T., Nishimura, M., *et al.* (1986) Preventive effects of cyclosporin on diabetes in NOD mice. *Diabetologia* **29:** 244–247.

Morkowski, S., Goldrath, A. W., Eastman, S., Ramachandra, L., Freed, D. C., Whiteley, P. and Rudensky, A. Y. (1995) T cell recognition of major histocompatibility class II complexes with invariant chain processing intermediates. *J. Exp. Med.* **182:** 1403–1413.

Morkowski, S., Raposo, G., Kleijimeer, M., Geuze, H. J. and Rudensky, A. Y. (1997) Assembly of an abundant endogenous major histocompatibility complex class II/peptide complex in class II compartments. *Eur. J. Immunol.* **27:** 609–617.

Morris, P., Shaman, J., Attaya, M., Amaya, M., Goodman, S., Bergman, C., Monaco, J. J. and Mellins, E. (1994) An essential role for HLA-DM in antigen presentation by class II major histocompatibilty molecules. *Nature* **368:** 551–554.

Morse, H. C. I. (1996) Molecular mimicry: CLIP, MHC class II supermotifs and MAIDS. *Immunol. Today* **17:** 47–48.

Mosyak, L., Zaller, D. M. and Wiley, D. C. (1998) The structure of HLA-DM, the peptide exchange catalyst that loads antigen onto class II MHC molecules during antigen presentation. *Immunity* **9:** 377–383.

Motta, A., Bremnes, B., Morelli, M. A., Frank, R. W., Saviano, G. and Bakke, O. (1995) Structure-activity relationship of the leucine-based sorting motifs in the cytosolic tail of the major histocompatibility complex-associated invariant chain. *J. Biol. Chem.* **270:** 27165–27171.

Motta, A., Amodeo, P., Fucile, P., Castiglione Morelli, M. A., Bremnes, B. and Bakke, O. (1997) A new triple-stranded α-helical bundle in solution: the assembling of the cytosolic tail of MHC-associated invariant chain. *Structure* **5:** 1453–1464.

Mouritsen, S., Hansen, A. S., Petersen, B. L. and Buus, S. (1992) pH dependence of the interaction between immunogenic peptides and MHC class II molecules. *J. Immunol.* **148:** 1438–1444.

Mozes, E., Dayan, M., Zisman, E., Brocke, S., Licht, A. and Pecht, I. (1989) Direct binding of a myasthenia gravis-related epitope to MHC class II molecules on living murine antigen-presenting cells. *EMBO J.* **8:** 4049–4052.

Muller, K. P., Schumacher, J. and Kyewski, B. A. (1993) Half-life of antigen/major histocompatibility complex class II complexes in vivo: intra- and interorgan variations. *Eur. J. Immunol.* **23:** 3203–3207.

Murray, J. S., Madri, J., Pasqualini, T. and Bottomly, K. (1993) Functional CD4 T cell subset interplay in an intact immune system. *J. Immunol.* **150:** 4270–4276.

Murray, J. S., Ferrandis-Edwards, D., Wolfe, C. J. and Schountz, T. (1994) Major histocompatibility complex regulation of T helper functions mapped to a peptide C terminus that controls ligand density. *Eur. J. Immunol.* **24:** 2337–2344.

Nabavieh, A., Chou, H., Volokhov, I., Lee, J. E., Purdy, L. E., Elliott, J. F., Singh, B. and Madrenas, J. (1998) Development of an I-A^{g7}-expressing antigen-presenting cell line: intrinsic molecular defect in compact I-A^{g7} dimer generation. *J. Autoimmun.* **11:** 63–71.

Nadimi, F., Moreno, J., Momburg, F., Heuser, A., Fuchs, S., Adorini, L. and Hämmerling, G. J. (1991) Antigen presentation of hen egg-white lysozyme but not of ribonuclease A is augmented by the major histocompatibility complex class II-associated invariant chain. *Eur. J. Immunol.* **21:** 1255–1263.

Nakagawa, T., Roth, W., Wong, P., Nelson, A., Farr, A., Deussing, J., Villadangos, J. A., Ploegh, H., Peters, C. and Rudensky, A. Y. (1998) Cathepsin L: critical role in Ii degradation and CD4 T cell selection in the thymus. *Science* **280:** 450–453.

Nakagawa, T. Y., Brissette, W. H., Lira, P. D., Griffiths, R. J., Petrushova, N., Stock, J., McNeish, J. D., Eastman, S. E., Howard, E. D., Clarke, S. R., *et al.* (1999) Impaired invariant chain degradation and antigen presentation and diminished collagen-induced arthritis in cathepsin S null mice. *Immunity* **10:** 207–217.

Nakano, N., Kikutani, H., Nishimoto, H. and Kishimoto, T. (1991) T cell receptor V gene usage of islet β cell-reactive T cells is not restricted in non-obese diabetic mice. *J. Exp. Med.* **173:** 1091–1097.

Nakhooda, A. F., Like, A. A., Chappel, C. I., Murray, F. T. and Marliss, E. B. (1977) The spontaneously diabetic Wistar rat. Metabolic and morphologic studies. *Diabetes* **26:** 100–112.

Nalefski, E. A., Shaw, K. T. Y. and Rao, A. (1995) An ion pair in class II major histocompatibility complex heterodimers critical for surface expression and peptide presentation. *J. Biol. Chem.* **270:** 22352–22360.

Naquet, P., Ellis, J., Tibensky, D., Kenshole, A., Singh, B., Hodges, R. and Delovitch, T. L. (1988) T cell autoreactivity to insulin in diabetic and related non-diabetic individuals. *J. Immunol.* **140:** 2569–2578.

Natarajan, S. K., Assadi, M. and Sadegh-Nasseri, S. (1999a) Stable peptide binding to MHC class II molecule is rapid and is determined by a receptive conformation shaped by prior association with low affinity peptides. *J. Immunol.* **162:** 4030–4036.

Natarajan, S. K., Stern, L. J. and Sadegh-Nasseri, S. (1999b) Sodium dodecyl sulfate stability of HLA-DR1 complexes correlates with burial of hydrophobic residues in pocket 1. *J. Immunol.* **162:** 3463–3470.

Naujokas, M. F., Morin, M., Anderson, M. S., Peterson, M. and Miller, J. (1993) The chondroitin sulfate form of invariant chain can enhance stimulation of T cell responses through interaction with CD44. *Cell* **74:** 257–268.

Naujokas, M. F., Arneson, L. S., Fineschi, B., Peterson, M. E., Sitterding, S., Hammond, A. T., Reilly, C., Lo, D. and Miller, J. (1995) Potent effects of low levels of MHC class II-associated invariant chain on $CD4^+$ T cell development. *Immunity* **3:** 359–372.

Naujokas, M. F., Southwood, S., Mathies, S. J., Appella, E., Sette, A. and Miller, J. (1998) T cell recognition of flanking residues of murine invariant chain-derived CLIP peptide bound to MHC class II. *Cell. Immunol.* **188:** 49–54.

Neefjes, J. (1999) CIIV, MIIC and other compartments for MHC class II loading. *Eur. J. Immunol.* **29:** 1421–1425.

Neefjes, J. J., Stollorz, V., Peters, P. J., Geuze, H. J. and Ploegh, H. L. (1990) The biosynthetic pathway of MHC class II but not class I molecules intersects the endocytic route. *Cell* **61:** 171–183.

Nelson, C. A., Roof, R. W., McCourt, D. W. and Unanue, E. R. (1992) Identification of the naturally-processed form of hen egg white lysozyme bound to the murine major histocompatibility complex class II molecule $I\text{-}A^k$. *Proc. Natl Acad. Sci. USA* **89:** 7380–7383.

Nelson, C. A., Petzold, S. J. and Unanue, E. R. (1994) Peptides determine the lifespan of MHC class II molecules in the antigen-presenting cell. *Nature* **371:** 250–252.

Nelson, C. A., Viner, N. J., Young, S. P., Petzold, S. J. and Unanue, E. R. (1996) A negatively charged anchor residue promotes high affinity binding to the MHC class II molecule I-A^k. *J. Immunol.* **157:** 755–762.

Nepom, G. T. (1990) A unified hypothesis for the complex genetics of HLA associations with IDDM. *Diabetes* **39:** 1153–1157.

Nepom, G. T. and Erlich, H. (1991) MHC class II molecules and autoimmunity. *Annu. Rev. Immunol.* **9:** 493–525.

Nepom, G. T. and Kwok, W. W. (1998) Molecular basis for HLA-DQ associations with IDDM. *Diabetes* **47:** 1177–1184.

Nerup, J., Mandrup-Poulsen, T., Molvig, J., Helqvist, S., Wogensen, L. and Egeberg, J. (1988) Mechanisms of pancreatic β-cell destruction in type I diabetes. *Diabetes Care* **11** (Suppl 1): 16–23.

Newcomb, J. R. and Cresswell, P. (1993) Characterisation of endogenous peptides bound to purified HLA-DR molecules and their absence from invariant chain-associated α β dimers. *J. Immunol.* **150:** 499–507.

Newton-Nash, D. K. and Eckels, D. D. (1993) Differential effect of polymorphism at HLA-DR1 β-chain positions 85 and 86 on binding and recognition of DR1-restricted antigenic peptides. *J. Immunol.* **150:** 1813–1821.

Nguyen, Q. V., Knapp, W. and Humphreys, R. E. (1989) Inhibition by leupeptin and antipain of the intracellular proteolysis of Ii. *Hum. Immunol.* **24:** 153–163.

Niimi, M., Nakai, Y. and Aida, Y. (1996) Identification of bovine invariant chain (Ii) gene by nucleotide sequencing. *Biochem. Biophys. Res. Comm.* **222:** 7–12.

Nijman, H. W., Kleijmeer, M. J., Ossevoort, M. A., Oorschot, V. M., Vierboom, M. P., van de Keur, M., Kenemans, P., Kast, W. M., Geuze, H. J. and Melief, C. J. (1995) Antigen capture and major histocompatibility class II compartments of freshly isolated and cultured human blood dendritic cells. *J. Exp. Med.* **182:** 163–174.

Nishimoto, H., Kikutani, H., Yamamura, K. and Kishimoto, T. (1987) Prevention of autoimmune insulitis by expression of I-E molecules in NOD mice. *Nature* **328:** 432–434.

Nizetic, D., Figueroa, F., Dembic, Z., Nevo, E. and Klein, J. (1987) Major histocompatibility complex gene organization in the mole rat *Spalax ehrenbergi*: evidence for transfer of function between class II genes. *Proc. Natl Acad. Sci. USA* **84:** 5828–5832.

Noorchashm, H., Noorchashm, N., Kern, J., Rostami, S. Y., Barker, C. F. and Naji, A. (1997) B-cells are required for the initiation of insulitis and sialitis in nonobese diabetic mice. *Diabetes* **46:** 941–946.

Noorchashm, H., Lieu, Y. K., Noorchashm, N., Rostami, S. Y., Greeley, S. A., Schlachterman, A., Song, H. K., Noto, L. E., Jevnikar, A. M., Barker, C. F., *et al.* (1999) I-A^{g7}-mediated antigen presentation by B lymphocytes is critical in overcoming a checkpoint in T cell tolerance to islet β cells of nonobese diabetic mice. *J. Immunol.* **163:** 743–750.

Nuchtern, J. G., Biddison, W. E. and Klausner, R. D. (1990) Class II MHC molecules can use the endogenous pathway of antigen presentation. *Nature* **343:** 74–76.

Nydam, T., Wade, T. K., Yadati, S., Gabriel, J. L., Barisas, B. G. and Wade, W. F. (1998) Mutations in MHC class II dimer of dimers contact residues: effects on antigen presentation. *Int. Immunol.* **10:** 1237–1249.

O'Donnell, B. F., O'Neill, C. M., Francis, D. M., Niimi, N., Barr, R. M., Barlow, R. J., Kobza Black, A., Welsh, K. I. and Greaves, M. W. (1999) Human leucocyte antigen class II associations in chronic idiopathic urticaria. *Br. J. Dermatol.* **140:** 853–858.

O'Sullivan, D. M., Noonan, D. and Quaranta, V. (1987) Four Ia invariant chain forms derive from a single gene by alternate splicing and alternate initiation of transcription/translation. *J. Exp. Med.* **166:** 444–460.

Odorizzi, C. G., Trowbridge, I. S., Xue, L., Hopkins, C. R., Davis, C. D. and Collawn, J. F. (1994) Sorting signals in the MHC class II invariant chain cytoplasmic tail and transmembrane region determine trafficking to an endocytic processing compartment. *J. Cell Biol.* **126:** 317–330.

Ogawa, M., Maruyama, T., Hasegawa, T., Kanaya, T., Kobayashi, F., Tochino, Y. and Uda, H. (1985) The inhibitory effect of neonatal thymectomy on the incidence of insulitis in non-obese (NOD) mice. *Biomed. Res.* **6:** 103–105.

Ogrinc, T., Dolenc, I., Ritonja, A. and Turk, V. (1993) Purification of the complex of cathepsin L and the MHC class II- associated invariant chain fragment from human kidney. *FEBS Lett.* **336:** 555–559.

Oi, V. T., Jones, P. P., Goding, J. W., Herzenberg, L. A. and Herzenberg, L. A. (1978) Properties of monoclonal antibodies to mouse Ig allotypes, H-2, and Ia antigens. *Curr. Top. Micro. Immunol.* **81:** 115–128.

Oiso, M., Nishi, T., Ishikawa, T., Nishimura, Y. and Matsushita, S. (1997) Differential binding of peptides substituted at putative C-terminal anchor residue to HLA-DQ8 and DQ9 differing only at β57. *Hum. Immunol.* **52:** 47–53.

Oiso, M., Matsushita, S., Nishi, T., Ishikawa, T., Nakano, N., Yoshida, K., Kikutani, H. and Nishimura, Y. (1998) Differential binding of peptides substituted at a putative C-terminal anchor residue to I-$A^{\beta 56His/\beta 57Ser}$ and I-$A^{\beta 56Pro/\beta 57Asp}$. *Immunogenetics* **47:** 411–414.

Oldstone, M. B. (1988) Prevention of type I diabetes in nonobese diabetic mice by virus infection. *Science* **239:** 500–502.

Oldstone, M. B., Nerenberg, M., Southern, P., Price, J. and Lewicki, H. (1991) Virus infection triggers insulin-dependent diabetes mellitus in a transgenic model: role of anti-self (virus) immune response. *Cell* **65:** 319–331.

Olerup, O., Aldener-Cannava, A., Fogdell-Hahn, A., Getty, R. R., Wagenknecht, D. R. and McIntyre, J. A. (1997) DQB1*0202 and the new DQB1*0203 allele: a fourth pair of DQB1 alleles differing only at codon 57. *Tissue Antigens* **49:** 271–273.

Ong, G. L., Goldenberg, D. M., Hansen, H. J. and Mattes, M. J. (1999) Cell-surface expression and metabolism of major histocompatibility complex class II invariant chain (CD74) by diverse cell lines. *Immunology* **98:** 296–302.

Otley, C. C., Wenstrup, R. J. and Hall, R. P. (1991) DNA sequence analysis and restriction fragment length polymorphism (RFLP) typing of the HLA-DQw2 alleles associated with dermatitis herpetiformis. *J. Invest. Dermatol.* **97:** 318–322.

Oukka, M., Andre, P., Turmel, P., Besnard, N., Angevin, V., Karlsson, L., Trans, P. L., Charron, D., Bihain, B., Kosmatopoulos, K., *et al.* (1997) Selectivity of the major histocompatibility

complex class II presentation pathway of cortical thymic epithelial cell lines. *Eur. J. Immunol.* **27:** 855–859.

Oxenius, A., Bachmann, M. F., Mathis, D., Benoist, C., Zinkernagel, R. M. and Hengartner, H. (1997) Functional in vivo MHC class II loading by endogenously synthesised glycoprotein during viral infection. *J. Immunol.* **158:** 5717–5726.

Ozato, K., Mayer, N. and Sachs, D. H. (1980) Hybridoma cell lines secreting monoclonal antibodies to mouse H-2 and Ia antigens. *J. Immunol.* **124:** 533–540.

Paliard, X., West, S. G., Lafferty, J. A., Clements, J. R., Kappler, J. W., Marrack, P. and Kotzin, B. L. (1991) Evidence for the effects of a superantigen in rheumatoid arthritis. *Science* **253:** 325–329.

Palmer, J. P., Asplin, C. M., Clemons, P., Lyen, K., Tatpati, O., Raghu, P. K. and Paquette, T. L. (1983) Insulin antibodies in insulin-dependent diabetics before insulin treatment. *Science* **222:** 1337–1339.

Pamer, E. and Cresswell, P. (1998) Mechanisms of MHC class I-restricted antigen processing. *Annu. Rev. Immunol.* **16:** 323–358.

Parish, N. M., Acha-Orbea, H., Simpson, E., Qin, S. X., Lund, T. and Cooke, A. (1993a) A comparative study of T-cell receptor Vβ usage in non-obese diabetic (NOD) and I-E transgenic NOD mice. *Immunology* **78:** 606–610.

Parish, N. M., Chandler, P., Quartey-Papafio, R., Simpson, E. and Cooke, A. (1993b) The effect of bone marrow and thymus chimerism between non-obese diabetic (NOD) and NOD-E transgenic mice, on the expression and prevention of diabetes. *Eur. J. Immunol.* **23:** 2667–2675.

Park, S.-J., Sadegh-Nasseri, S. and Wiley, D. C. (1995) Invariant chain made in *Escherichia coli* has an exposed N-terminal segment that blocks antigen binding to HLA-DR1 and a trimeric C-terminal segment that binds empty HLA-DR1. *Proc. Natl Acad. Sci. USA* **92:** 11289–11293.

Partanen, J., Peterson, P., Westman, P., Aranko, S. and Krohn, K. (1994) Major histocompatibility complex class II and III in Addison's disease. MHC alleles do not predict autoantibody specificity and 21-hydroxylase gene polymorphism has no independent role in disease susceptibility. *Hum. Immunol.* **41:** 135–140.

Paul, W. E. and Seder, R. A. (1994) Lymphocyte responses and cytokines. *Cell* **76:** 241–251.

Pauling, L., Corey, R. B. and Branson, H. R. (1951) The structure of proteins: Two hydrogen-bonded helical conformations of the polypeptide chain. *Proc. Natl Acad. Sci. USA* **37:** 205–211.

Pedrazzini, T., Sette, A., Albertson, M. and Grey, H. M. (1991) Free ligand-induced dissociation of MHC-antigen complexes. *J. Immunol.* **146:** 3496–3501.

Pernis, B. (1985) Internalisation of lymphocyte membrane components. *Immunol. Today* **6:** 45–49.

Peters, P. J., Neefjes, J. J., Oorschot, V., Ploegh, H. L. and Geuze, H. J. (1991) Segregation of MHC class II molecules from MHC class I molecules in the Golgi complex for transport to lysosomal compartments. *Nature* **349:** 669–676.

Peters, P. J., Raposo, G., Neefjes, J. J., Oorschot, V., Leijendekker, R. L., Geuze, H. J. and Ploegh, H. L. (1995) Major histocompatibility complex class II compartments in human B lymphoblastoid cells are distinct from early endosomes. *J. Exp. Med.* **182:** 325–334.

Peterson, M. and Miller, J. (1992) Antigen presentation enhanced by the alternatively spliced invariant chain gene product p41. *Nature* **357:** 596–598.

Peterson, M. and Sant, A. J. (1998) The inability of the nonobese diabetic class II molecule to form stable peptide complexes does not reflect a failure to interact productively with DM. *J. Immunol.* **161:** 2961–2967.

Petersen, J. S., Karlsen, A. E., Markholst, H., Worsaae, A., Dyrberg, T. and Michelsen, B. (1994) Neonatal tolerisation with glutamic acid decarboxylase but not with bovine serum albumin delays the onset of diabetes in NOD mice. *Diabetes* **43:** 1478–1484.

Pfeifer, J. D., Wick, M. J., Roberts, R. L., Findlay, K., Normark, S. J. and Harding, C. V. (1993) Phagocytic processing of bacterial antigens for class I MHC presentation to T cells. *Nature* **361:** 359–362.

Pfeiffer, C., Stein, J., Southwood, S., Ketelaar, H., Sette, A. and Bottomly, K. (1995) Altered peptide ligands can control CD4 T lymphocyte differentiation in vivo. *J. Exp. Med.* **181:** 1569–1574.

Pierre, P. and Mellman, I. (1998) Developmental regulation of invariant chain proteolysis controls MHC class II trafficking in mouse dendritic cells. *Cell* **93:** 1135–1145.

Pieters, J., Bakke, O. and Dobberstein, B. (1993) The MHC class II-associated invariant chain contains two endosomal targeting signals within its cytoplasmic tail. *J. Cell Sci.* **106:** 831–846.

Piganelli, J. D., Martin, T. and Haskins, K. (1998) Splenic macrophages from the NOD mouse are defective in the ability to present antigen. *Diabetes* **47:** 1212–1218.

Pilström, B. and Böhme, J. (1997) Alleviation of insulitis in NOD mice is associated with expression of transgenic MHC E molecules on primary antigen-presenting cells. *Immunology* **90:** 483–488.

Pilström, B., Björk, L. and Böhme, J. (1995) Demonstration of a TH1 cytokine profile in the late phase of NOD insulitis. *Cytokine* **7:** 806–814.

Pinet, V. M. and Long, E. O. (1998) Peptide loading onto recycling HLA-DR molecules occurs in early endosomes. *Eur J Immunol* **28:** 799-804.

Pinet, V., Malnati, M. S. and Long, E. O. (1994) Two processing pathways for the MHC class II-restricted presentation of exogenous influenza virus antigen. *J. Immunol.* **152:** 4852–4860.

Pinet, V., Vergelli, M., Martin, R., Bakke, O. and Long, E. O. (1995) Antigen presentation mediated by recycling of surface HLA-DR molecules. *Nature* **375:** 603–606.

Pious, D., Dixon, L., Levine, F., Cotner, T. and Johnson, R. (1985) HLA class II regulation and structure: Analysis with HLA-DR3 and HLA-DP point mutants. *J. Exp. Med.* **162:** 1193–1207.

Podolin, P. L., Pressey, A., DeLarato, N. H., Fischer, P. A., Peterson, L. B. and Wicker, L. S. (1993) I-E$^+$ nonobese diabetic mice develop insulitis and diabetes. *J. Exp. Med.* **178:** 793–803.

Poirier, G. and Chain, B. (1993) Analysis of the cellular requirements for the binding of exogenous peptides to MHC class II molecules. *Cell. Immunol.* **152:** 456–467.

Pond, L. and Watts, C. (1997) Characterisation of transport of newly-assembled, T cell-stimulatory MHC class II-peptide complexes from MHC class II compartments to the cell surface. *J. Immunol.* **159:** 543–553.

Pond, L. and Watts, C. (1999) Functional early endosomes are required for maturation of major histocompatibility complex class II molecules in human B lymphoblastoid cells. *J. Biol. Chem.* **274:** 18049–18054.

Pond, L., Kuhn, L. A., Teyton, L., Schutze, M.-P., Tainer, J. A., Jackson, M. R. and Peterson, P. A. (1995) A role for acidic residues in di-leucine motif-based targeting to the endocytic pathway. *J. Biol. Chem.* **270:** 19989–19997.

Ponzoni, M., Guarnaccia, F., Corrias, M. V. and Cornaglia-Ferraris, P. (1993) Uncoordinate induction and differential regulation of HLA class-I and class-II expression by γ-interferon in differentiating human neuroblastoma cells. *Int. J. Cancer.* **55:** 817–823.

Pozzilli, P., Signore, A., Williams, A. J. and Beales, P. E. (1993) NOD mouse colonies around the world: Recent facts and figures. *Immunol. Today* **14:** 193–196.

Prochazka, M., Serreze, D. V., Worthen, S. M. and Leiter, E. H. (1989) Genetic control of diabetogenesis in NOD/Lt mice. Development and analysis of congenic stocks. *Diabetes* **38:** 1446–1455.

Pugliese, A. and Eisenbarth, G. S. (1996) Human type I diabetes mellitus: Genetic susceptibility and resistance. *In:* Eisenbarth, G. S. and Lafferty, K. J. (*eds.*), Type I diabetes: Molecular, cellular and clinical immunology. Oxford University Press, New York.

Pugliese, A., Solimena, M., Awdeh, Z. L., Alper, C. A., Bugawan, T., Erlich, H. A., De Camilli, P. and Eisenbarth, G. S. (1993) Association of HLA-DQB1*0201 with stiff-man syndrome. *J. Clin. Endocrinol. Metab.* **77:** 1550–1553.

Pugliese, A., Gianani, R., Moromisato, R., Awdeh, Z. L., Alper, C. A., Erlich, H. A., Jackson, R. A. and Eisenbarth, G. S. (1995) HLA-DQB1*0602 is associated with dominant protection from diabetes even among islet cell antibody-positive first-degree relatives of patients with IDDM. *Diabetes* **44:** 608–613.

Pujol-Borrell, R., Todd, I., Doshi, M., Bottazzo, G. F., Sutton, R., Gray, D., Adolf, G. R. and Feldmann, M. (1987) HLA class II induction in human islet cells by interferon-gamma plus tumour necrosis factor or lymphotoxin. *Nature* **326:** 304–306.

Pukel, C., Baquerizo, H. and Rabinovitch, A. (1988) Destruction of rat islet cell monolayers by cytokines. Synergistic interactions of interferon-gamma, tumor necrosis factor, lymphotoxin, and interleukin 1. *Diabetes* **37:** 133–136.

Pullen, A. M., Marrack, P. and Kappler, J. W. (1988) The T-cell repertoire is heavily influenced by tolerance to polymorphic self-antigens. *Nature* **335:** 796–801.

Qiu, Y., Wandinger-Ness, A., Dalke, D. P. and Pierce, S. (1994) Separation of subcellular compartments containing distinct functional forms of MHC class II. *J. Cell Biol.* **125:** 595–605.

Quaratino, S., Thorpe, C. J., Travers, P. J. and Londei, M. (1995) Similar antigenic surfaces, rather than sequence homology, dictate T-cell epitope molecular mimicry. *Proc. Natl Acad. Sci. USA* **92:** 10398–10402.

Quarsten, H., Paulsen, G., Johansen, B. H., Thorpe, C. J., Holm, A., Buus, S. and Sollid, L. M. (1998) The P9 pocket of HLA-DQ2 (non-Aspβ57) has no particular preference for negatively-charged anchor residues found in other type 1 diabetes-predisposing non-Aspβ57 MHC class II molecules. *Int. Immunol.* **10:** 1229–1236.

Quartey-Papafio, R., Lund, T., Chandler, P., Picard, J., Ozegbe, P., Day, S., Hutchings, P. R., O'Reilly, L., Kioussis, D., Simpson, E., *et al.* (1995) Aspartate at position 57 of nonobese diabetic I-A^{g7} β-chain diminishes the spontaneous incidence of insulin-dependent diabetes mellitus. *J. Immunol.* **154:** 5567–5575.

Rabinovitch, A. (1994) Immunoregulatory and cytokine imbalances in the pathogenesis of IDDM. Therapeutic intervention by immunostimulation? *Diabetes* **43:** 613–621.

Rabinovitch, A., Sumoski, W., Rajotte, R. V. and Warnock, G. L. (1990) Cytotoxic effects of cytokines on human pancreatic islet cells in monolayer culture. *J. Clin. Endocrinol. Metab.* **71:** 152–156.

Rabinovitch, A., Suarez, W. L. and Power, R. F. (1992) Combination therapy with an antioxidant and a corticosteroid prevents autoimmune diabetes in NOD mice. *Life Sci.* **51:** 1937–1943.

Rabinowitz, J. D., Vrljic, M., Kasson, P. M., Liang, M. N., Busch, R., Boniface, J. J., Davis, M. M. and McConnell, H. M. (1998) Formation of a highly peptide-receptive state of class II MHC. *Immunity* **9:** 699–709.

Raddrizzani, L., Sturniolo, T., Guenot, J., Bono, E., Gallazzi, F., Nagy, Z. A., Sinigaglia, F. and Hammer, J. (1997) Different modes of peptide interaction enable HLA-DQ and HLA-DR molecules to bind diverse peptide repertoires. *J. Immunol.* **159:** 703–711.

Ramachandra, L., Kovats, S., Eastman, S. and Rudensky, A. Y. (1996) Variation in HLA-DM expression influences conversion of MHC class II αβ:class II-associated invariant chain peptide complexes to mature peptide-bound class II αβ dimers in a normal B cell line. *J. Immunol.* **156:** 2196–2204.

Rammensee, H.-G., Friede, T. and Stevanovic, S. (1995) MHC ligands and peptide motifs: first listing. *Immunogenetics* **41:** 178–228.

Ramsdell, F., Seaman, M. S., Miller, R. E., Picha, K. S., Kennedy, M. K. and Lynch, D. H. (1994) Differential ability of Th1 and Th2 T cells to express Fas ligand and to undergo activation-induced cell death. *Int. Immunol.* **6:** 1545–1553.

Rapoport, M. J., Jaramillo, A., Zipris, D., Lazarus, A. H., Serreze, D. V., Leiter, E. H., Cyopick, P., Danska, J. S. and Delovitch, T. L. (1993a) Interleukin 4 reverses T cell proliferative unresponsiveness and prevents the onset of diabetes in nonobese diabetic mice. *J. Exp. Med.* **178:** 87–99.

Rapoport, M. J., Lazarus, A. H., Jaramillo, A., Speck, E. and Delovitch, T. L. (1993b) Thymic T cell anergy in autoimmune nonobese diabetic mice is mediated by deficient T cell receptor regulation of the pathway of p21ras activation. *J. Exp. Med.* **177:** 1221–1226.

Raposo, G., Nijman, H. W., Stoorvogel, W., Liejendekker, R., Harding, C. V., Melief, C. J. and Geuze, H. J. (1996) B lymphocytes secrete antigen-presenting vesicles. *J. Exp. Med.* **183:** 1161–1172.

Rashba, E. J., Reich, E. P., Janeway, C. A. and Sherwin, R. S. (1993) Type 1 diabetes mellitus: an imbalance between effector and regulatory T cells? *Acta Diabetol.* **30:** 61–69.

Reay, P. A., Wettstein, D. A. and Davis, M. M. (1992) pH dependence and exchange of high and low responder peptides binding to a class II MHC molecule. *EMBO J* **11:** 2829–2839.

Reay, P. A., Kantor, R. M. and Davis, M. M. (1994) Use of global amino acid replacements to define the requirements for MHC binding and T cell recognition of moth cytochrome *c* (93–103). *J. Immunol.* **152:** 3946–3957.

Reddy, S., Bibby, N. J. and Elliott, R. B. (1988) Ontogeny of islet cell antibodies, insulin autoantibodies and insulitis in the non-obese diabetic mouse. *Diabetologia* **31:** 322–328.

Reed, A. M., Collins, E. J., Shock, L. P., Klapper, D. G. and Frelinger, J. A. (1997) Diminished class II-associated Ii peptide binding to the juvenile dermatomyositis HLA-DQα1*0501/DQβ1*0301 molecule. *J. Immunol.* **159:** 6260–6265.

Reich, E.-P., Sherwin, R. S., Kanagawa, O. and Janeway, C. A., Jr. (1989) An explanation for the protective effect of the MHC class II I-E molecule in murine diabetes. *Nature* **341:** 326–328.

Reich, E.-P., Sherwin, R. S., Kanagawa, O. and Janeway, C. A., Jr. (1991) An explanation for the protective effect of the MHC class II I-E molecule in murine diabetes [Correction]. *Nature* **352:** 88.

Reich, E.-P., von Grafenstein, H., Barlow, A., Swenson, K. E., Williams, K. and Janeway, C. A. (1994) Self peptides isolated from MHC glycoproteins of non-obese diabetic mice. *J. Immunol.* **152:** 2279–2288.

Reich, Z., Altman, J. D., Boniface, J. J., Lyons, D. S., Kozono, H., Ogg, G., Morgan, C. and Davis, M. M. (1997) Stability of empty and peptide-loaded class II major histocompatibility complex molecules at neutral and endosomal pH: Comparison to class I proteins. *Proc. Natl Acad. Sci. USA* **94:** 2495–2500.

Reid, P. A. and Watts, C. (1990) Cycling of cell-surface MHC glycoproteins through primaquine-sensitive intracellular compartments. *Nature* **346:** 655–657.

Reizis, B., Mor, F., Eisenstein, M., Schild, H., Stefanovic, S., Rammensee, H. G. and Cohen, I. R. (1996) The peptide binding specificity of the MHC class II I-A molecule of the Lewis rat, RT1.B^{l}. *Int. Immunol.* **8:** 1825–1832.

Reizis, B., Altmann, D. M. and Cohen, I. R. (1997a) Biochemical characterisation of the human diabetes-associated HLA-DQ8 allelic product: similarity to the major histocompatibility complex class II I-A^{g7} protein of non-obese diabetic mice. *Eur. J. Immunol.* **27:** 2478–2483.

Reizis, B., Eisenstein, M., Bockova, J., Konen-Waisman, S., Mor, F., Elias, D. and Cohen, I. R. (1997b) Molecular characterisation of the diabetes-associated mouse MHC class II protein, I-A^{g7}. *Int. Immunol.* **9:** 43–51.

Reizis, B., Eisenstein, M., Mor, F. and Cohen, I. R. (1998) The peptide-binding strategy of the MHC class II I-A molecules. *Immunol. Today* **19:** 212–216.

Rellahan, B. L., Jones, L. A., Kruisbeek, A. M., Fry, A. M. and Matis, L. A. (1990) In vivo induction of anergy in peripheral Vβ8^{+} T cells by staphylococcal enterotoxin B. *J. Exp. Med.* **172:** 1091–1100.

Renno, T. and Acha-Orbea, H. (1996) Superantigens in autoimmune diseases: still more shades of gray. *Immunol. Rev.* **154:** 175–191.

Reske, K. and Weitzel, R. (1985) Immunologically discrete conformation isomers of I-A locus-equivalent class II molecules detected in Lewis rats. *Eur. J. Immunol.* **15:** 1229–1239.

Reyes, V. E., Lu, S. and Humphreys, R. E. (1991) Cathepsin B cleavage of Ii from class II MHC α- and β-chains. *J. Immunol.* **146:** 3877–3880.

Riberdy, J. M., Newcomb, J. R., Surman, M. J., Barbosa, J. A. and Cresswell, P. (1992) HLA-DR molecules from an antigen-processing mutant cell line are associated with invariant chain peptides. *Nature* **360:** 474–477.

Richards, J. E., Pravtcheva, D. D., Day, C., Ruddle, F. H. and Jones, P. P. (1985) Murine invariant chain gene: chromosomal assignment and segregation in recombinant inbred strains. *Immunogenetics* **22:** 193–199.

Ridgway, W. M., Fasso, M., Lanctot, A., Garvey, C. and Fathman, C. G. (1996) Breaking self-tolerance in nonobese diabetic mice. *J. Exp. Med.* **183:** 1657–1662.

Ridgway, W. M. and Fathman, C. G. (1998) The association of MHC with autoimmune diseases: understanding the pathogenesis of autoimmune diabetes. *Clin. Immunol. Immunopathol.* **86:** 3–10.

Riese, R. J., Wolf, P. R., Bromme, D., Natkin, L. R., Villadangos, J. A., Ploegh, H. L. and Chapman, H. A. (1996) Essential role for cathepsin in MHC class II-associated invariant chain processing and peptide loading. *Immunity* **4:** 357–366.

Riese, R. J., Mitchell, R. N., Villadangos, J. A., Shi, G. P., Palmer, J. T., Karp, E. R., De Sanctis, G. T., Ploegh, H. L. and Chapman, H. A. (1998) Cathepsin S activity regulates antigen presentation and immunity. *J. Clin. Invest.* **101:** 2351–2363.

Riley, W. J., Maclaren, N. K., Krischer, J., Spillar, R. P., Silverstein, J. H., Schatz, D. A., Schwartz, S., Malone, J., Shah, S., Vadheim, C., *et al.* (1990) A prospective study of the development of diabetes in relatives of patients with insulin-dependent diabetes. *N. Engl. J. Med.* **323:** 1167–1172.

Rivero, V. E., Cailleau, C., Depiante-Depaoli, M., Riera, C. M. and Carnaud, C. (1998) Non-obese diabetic (NOD) mice are genetically susceptible to experimental autoimmune prostatitis (EAP). *J. Autoimmun.* **11:** 603–610.

Robbins, N. F., Hammond, C., Denzin, L. K., Pan, M. and Cresswell, P. (1996) Trafficking of major histocompatibility complex class II molecules through intracellular compartments containing HLA-DM. *Hum. Immunol.* **45:** 13–23.

Robinson, C. P., Yamachika, S., Bounous, D. I., Brayer, J., Jonsson, R., Holmdahl, R., Peck, A. B. and Humphreys-Beher, M. G. (1998) A novel NOD-derived murine model of primary Sjögren's syndrome. *Arthritis Rheum.* **41:** 150–156.

Roche, P. A. and Cresswell, P. (1990a) High-affinity binding of an influenza hemagglutinin-derived peptide to purified HLA-DR. *J. Immunol.* **144:** 1849–1856.

Roche, P. A. and Cresswell, P. (1990b) Invariant chain association with HLA-DR molecules inhibits immunogenic peptide binding. *Nature* **345:** 615–618.

Roche, P. A. and Cresswell, P. (1991) Proteolysis of the class II-associated invariant chain generates a peptide binding site in intracellular HLA-DR molecules. *Proc. Natl Acad. Sci. USA* **88:** 3150–3154.

Roche, P. A., Marks, M. S. and Cresswell, P. (1991) Formation of a nine-subunit complex by HLA class II glycoproteins and the invariant chain. *Nature* **354:** 392–394.

Roche, P. A., Teletski, C. L., Karp, D. R., Pinet, V., Bakke, O. and Long, E. O. (1992) Stable surface expression of invariant chain prevents peptide presentation by HLA-DR. *EMBO J.* **11:** 2841–2847.

Roche, P. A., Teletski, C. L., Stang, E., Bakke, O. and Long, E. O. (1993) Cell-surface HLA-DR-invariant chain complexes are targeted to endosomes by rapid internalisation. *Proc. Natl Acad. Sci. USA* **90:** 8581–8585.

Rock, K. L., Gamble, S. and Rothstein, L. (1990) Presentation of exogenous antigen with class I major histocompatibility complex molecules. *Science* **249:** 918–921.

Rodewald, H. R. and Fehling, H. J. (1998) Molecular and cellular events in early thymocyte development. *Adv. Immunol.* **69:** 1–112.

Roep, B. O. (1996) T-cell responses to autoantigens in IDDM. The search for the Holy Grail. *Diabetes* **45:** 1147–1156.

Rogers, P. R. and Croft, M. (1999) Peptide dose, affinity, and time of differentiation can contribute to the Th1/Th2 cytokine balance. *J. Immunol.* **163:** 1205–1213.

Romagnani, S. (1997) The Th1/ Th2 paradigm. *Immunol. Today* **18:** 263–266.

Romagnoli, P. and Germain, R. N. (1994) The CLIP region of invariant chain plays a critical role in regulating major histocompatibility complex class II folding, transport, and peptide occupancy. *J. Exp. Med.* **180:** 1107–1113.

Romagnoli, P. and Germain, R. N. (1995) Inhibition of invariant chain (Ii)-calnexin interaction results in enhanced degradation of Ii but does not prevent the assembly of αβIi complexes. *J. Exp. Med.* **182:** 2027–2036.

Romagnoli, P., Layet, C., Yewdell, J., Bakke, O. and Germain, R. N. (1993) Relationship between invariant chain expression and major histocompatibility complex class II transport into early and late endocytic compartments. *J. Exp. Med.* **177:** 583–596.

Rønningen, K. S. (1997) Genetics in the prediction of insulin-dependent diabetes mellitus: from theory to practice. *Ann. Med.* **29:** 387–392.

Rønningen, K. S., Iwe, T., Halstensen, T. S., Spurkland, A. and Thorsby, E. (1989) The amino acid at position 57 of the HLA-DQ β-chain and susceptibility to develop insulin-dependent diabetes mellitus. *Hum. Immunol.* **26:** 215–225.

Roosnek, E., Demotz, S., Corradin, G. and Lanzavecchia, A. (1988) Kinetics of MHC-antigen complex formation on antigen-presenting cells. *J. Immunol.* **140:** 4079–4082.

Rost, B. and Sander, C. (1993) Prediction of protein secondary structure at better than 70% accuracy. *J. Mol. Biol.* **232:** 584–599.

Rost, B. and Sander, C. (1994) Combining evolutionary information and neural networks to predict protein secondary structure. *Proteins* **19:** 55–77.

Rothbard, J. B. and Gefter, M. L. (1991) Interactions between immunogenic peptides and MHC proteins. *Annu. Rev. Immunol.* **9:** 527–565.

Rothbard, J. B., Busch, R., Howland, K., Bal, V., Fenton, C., Taylor, W. R. and R., L. J. (1989) Structural analysis of a peptide–HLA class II complex: identification of critical interactions for its formation and recognition by T cell receptor. *Int. Immunol.* **1:** 479–486.

Rotter, J. I. and Landaw, E. M. (1984) Measuring the genetic contribution of a single locus to a multilocus disease. *Clin. Genet.* **26:** 529–542.

Rötzschke, O. and Falk, K. (1994) Origin, structure and motifs of naturally processed MHC class II ligands. *Curr. Op. Immunol.* **6:** 45–51.

Roucard, C., Garban, F., Mooney, N. A., Charron, D. J. and Ericson, M. L. (1996) Conformation of human leukocyte antigen class II molecules. *J. Biol. Chem.* **271:** 13993–14000.

Rudensky, A. Y., Preston-Hurlburt, P., Hong, S.-C., Barlow, A. and Janeway, C. A. J. (1991) Sequence analysis of peptides bound to MHC class II molecules. *Nature* **353:** 622–627.

Rudensky, A. Y., Maric, M., Eastman, S., Shoemaker, L., DeRoos, P. C. and Blum, J. S. (1994) Intracellular assembly and transport of endogenous peptide-MHC class II complexes. *Immunity* **1:** 585–594.

Runnels, H. A., Moore, J. C. and Jensen, P. E. (1996) A structural transition in class II major histocompatibility complex proteins at mildly acidic pH. *J. Exp. Med.* **183:** 127–136.

Sadegh-Nasseri, S. and Germain, R. N. (1991) A role for peptide in determining MHC class II structure. *Nature* **353:** 167–170.

Sadegh-Nasseri, S. and McConnell, H. M. (1989) A kinetic intermediate in the reaction of an antigenic peptide and I-E^k. *Nature* **337:** 274–276.

Sadelain, M. W., Qin, H. Y., Lauzon, J. and Singh, B. (1990) Prevention of type I diabetes in NOD mice by adjuvant immunotherapy. *Diabetes* **39:** 583–589.

Saito, T., Kimura, M., Kawasaki, T., Sato, S. and Tomita, Y. (1997) MHC class II antigen-associated invariant chain on renal cell cancer may contribute to the anti-tumor immune response of the host. *Cancer Lett.* **115:** 121–127.

Salamero, J., Humbert, M., Cosson, P. and Davoust, J. (1990) Mouse B lymphocyte-specific endocytosis and recycling of MHC class II molecules. *EMBO J.* **9:** 3489–3496.

Salamov, A. A. and Solovyev, V. V. (1995) Prediction of protein secondary structure by combining nearest-neighbour algorithms and multiple sequence alignments. *J. Mol. Biol.* **247:** 11–15.

Salojin, K. V., Zhang, J., Madrenas, J. and Delovitch, T. L. (1998) T-cell anergy and altered T-cell receptor signaling: effects on autoimmune disease. *Immunol. Today* **19:** 468–473.

Samaan, A., Thibodeau, J., Mahana, W., Castellino, F., Cazenave, P. A. and Kindt, T. J. (1999) Cellular distribution of a mixed MHC class II heterodimer between DRα and a chimeric DOβ chain. *Int. Immunol.* **11:** 99–111.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular cloning: A laboratory manual., 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Samelson, L. E., Germain, R. N. and Schwartz, R. H. (1983) Monoclonal antibodies against the antigen receptor on a cloned T-cell hybrid. *Proc. Natl Acad. Sci. USA* **80:** 6972–6976.

Sanderson, F., Kleijmeer, M. J., Kelly, A., Verwoerd, D., Tulp, A., Neefjes, J. J., Geuze, H. J. and Trowsdale, J. (1994a) Accumulation of HLA-DM, a regulator of antigen presentation, in MHC class II compartments. *Science* **266:** 1566–1569.

Sanderson, F., Powis, S. H., Kelly, A. P. and Trowsdale, J. (1994b) Limited polymorphism in HLA-DM does not involve the peptide binding groove. *Immunogenetics* **39:** 56–58.

Sanderson, F., Thomas, C., Neefjes, J. and Trowsdale, J. (1996) Association between HLA-DM and HLA-DR *in vivo*. *Immunity* **4:** 87–96.

Sanjeevi, C. B., DeWeese, C., Landin-Olsson, M., Kockum, I., Dahlquist, G., Lernmark, A. and Lybrand, T. P. (1997) Analysis of critical residues of HLA-DQ6 molecules in insulin-dependent diabetes mellitus. *Tissue Antigens* **50:** 61–65.

Sant, A. J., Cullen, S. E. and Schwartz, B. D. (1985) Biosynthetic relationships of the chondroitin sulfate proteoglycan with Ia and invariant chain glycoproteins. *J. Immunol.* **135:** 416–422.

Santamaria, P., Boyce-Jacino, M. T., Lindstrom, A. L., Barbosa, J. J., Faras, A. J. and Rich, S. S. (1991) Detection of novel sequence heterogeneity and haplotypic diversity of HLA class II genes. *Immunogenetics* **33:** 374–387.

Santamaria, P., Barbosa, J. J., Lindstrom, A. L., Lemke, T. A., Goetz, F. C. and Rich, S. S. (1994) HLA-DQB1-associated susceptibility that distinguishes Hashimoto's thyroiditis from Graves' disease in type I diabetic patients. *J. Clin. Endocrinol. Metab.* **78:** 878–883.

Satoh, J., Seino, H., Abo, T., Tanaka, S., Shintani, S., Ohta, S., Tamura, K., Sawai, T., Nobunaga, T., Oteki, T., *et al.* (1989) Recombinant human tumor necrosis factor-α suppresses autoimmune diabetes in nonobese diabetic mice. *J. Clin. Invest.* **84:** 1345–1348.

Saudrais, C., Spehner, D., de la Salle, H., Bohbot, A., Cazenave, J. P., Goud, B., Hanau, D. and Salamero, J. (1998) Intracellular pathway for the generation of functional MHC class II peptide complexes in immature human dendritic cells. *J. Immunol.* **160:** 2597–2607.

Schafer, P. H. and Pierce, S. K. (1994) Evidence for dimers of MHC class II molecules in B lymphocytes and their role in low affinity T cell responses. *Immunity* **1:** 699–707.

Schafer, P. H., Green, J. M., Malapati, S., Gu, L. and Pierce, S. K. (1996) HLA-DM is present in one-fifth the amount of HLA-DR in the class II peptide-loading compartment where it associates with leupeptin-induced peptide (LIP)-HLA-DR complexes. *J. Immunol.* **157:** 5487–5495.

Schafer, P. H., Malapati, S., Hanfelt, K. K. and Pierce, S. K. (1998) The assembly and stability of MHC class II-$(\alpha\beta)_2$ superdimers. *J. Immunol.* **161:** 2307–2316.

Schägger, H. and von Jagow, G. (1987) Tricine-sodium dodecyl sulphate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* **166:** 368–379.

Schaiff, W. T., Hruska, K. A., Jr., Bono, C., Shuman, S. and Schwartz, B. D. (1991) Invariant chain influences post-translational processing of HLA-DR molecules. *J. Immunol.* **147:** 603–608.

Schaiff, W. T., Hruska, K. A., Jr., McCourt, D. W., Green, M. and Schwartz, B. D. (1992) HLA-DR associates with specific stress proteins and is retained in the endoplasmic reticulum in invariant chain negative cells. *J. Exp. Med.* **176:** 657–666.

Schenning, L., Larhammar, D., Bill, P., Wiman, K., Jonsson, A. K., Rask, L. and Peterson, P. A. (1984) Both α- and β-chains of HLA-DC class II histocompatibility antigens display extensive polymorphism in their amino-terminal domains. *EMBO J.* **3:** 447–452.

Schiffenbauer, J., Soos, J. and Johnson, H. (1998) The possible role of bacterial superantigens in the pathogenesis of autoimmune disorders. *Immunol. Today* **19:** 117–120.

Schild, H., Rotzschke, O., Kalbacher, H. and Rammensee, H. G. (1990) Limit of T cell tolerance to self proteins by peptide presentation. *Science* **247:** 1587–1589.

Schild, H., Grüneberg, U., Pougialis, G., Wallny, H.-J., Keilholz, W., Stevanovic, S. and Rammensee, H.-G. (1995) Natural ligand motifs of H-2E molecules are allele specific and illustrate homology to HLA-DR molecules. *Int. Immunol.* **7:** 1957–1965.

Schmid, S. L., Fuchs, R., Male, P. and Mellman, I. (1988) Two distinct subpopulations of endosomes involved in membrane recycling and transport to lysosomes. *Cell* **52:** 73–83.

Schmidt, D., Verdaguer, J., Averill, N. and Santamaria, P. (1997) A mechanism for the major histocompatibility complex-linked resistance to autoimmunity. *J. Exp. Med.* **186:** 1059–1075.

Schmidt, D., Amrani, A., Verdaguer, J., Bou, S. and Santamaria, P. (1999) Autoantigen-independent deletion of diabetogenic CD4$^+$ thymocytes by protective MHC class II molecules. *J. Immunol.* **162:** 4627–4636.

Scholl, P. R., Diez, A., Karr, R., Sekaly, R. P., Trowsdale, J. and Geha, R. S. (1990a) Effect of isotypes and allelic polymorphism on the binding of staphylococcal exotoxins to MHC class II molecules. *J. Immunol.* **144:** 226–230.

Scholl, P. R., Sekaly, R. P., Diez, A., Glimcher, L. H. and Geha, R. S. (1990b) Binding of toxic shock syndrome toxin-1 to murine major histocompatibility complex class II molecules. *Eur. J. Immunol.* **20:** 1911–1916.

Schreiber, K. L., Bell, M. P., Huntoon, C. J., Rajagopalan, S., Brenner, M. B. and McKean, D. J. (1994) Class II histocompatibility molecules associate with calnexin during assembly in the endoplasmic reticulum. *Int. Immunol.* **6:** 101–111.

Schröder, K., Martoglio, B., Hofmann, M., Hölscher, C., Hartmann, E., Prehn, S., Rapoport, T. A. and Dobberstein, B. (1999) Control of glycosylation of MHC class II-associated invariant chain by translocon-associated RAMP4. *EMBO J.* **18:** 4804–4815.

Schutze, M. P., Peterson, P. A. and Jackson, M. R. (1994) An N-terminal double-arginine motif maintains type II membrane proteins in the endoplasmic reticulum. *EMBO J.* **13:** 1696–1705.

Schwizer, R. W., Leiter, E. H. and Evans, R. (1984) Macrophage-mediated cytotoxicity against cultured pancreatic islet cells. *Transplantation* **37:** 539–544.

Scofield, R. H., Frank, M. B., Neas, B. R., Horowitz, R. M., Hardgrave, K. L., Fujisaku, A., McArthur, R. and Harley, J. B. (1994) Cooperative association of T cell β receptor and HLA-DQ alleles in the production of anti-Ro in systemic lupus erythematosus. *Clin. Immunol. Immunopathol.* **72:** 335–341.

Scott, C. A., Peterson, P. A., Teyton, L. and Wilson, I. A. (1998) Crystal structures of two I-A^d–peptide complexes reveal that high affinity can be achieved without large anchor residues. *Immunity* **8:** 319–329.

Sebzda, E., Mariathasan, S., Ohteki, T., Jones, R., Bachmann, M. F. and Ohashi, P. S. (1999) Selection of the T cell repertoire. *Annu. Rev. Immunol.* **17:** 829–874.

Seglen, P. O. and Bohley, P. (1992) Autophagy and other vacuolar protein degradation mechanisms. *Experientia* **48:** 158–172.

Sekaly, R. P., Jacobson, S., Richert, J. R., Tonnelle, C., McFarland, H. F. and Long, E. O. (1988) Antigen presentation to HLA class II-restricted measles virus-specific T-cell clones can occur in the absence of the invariant chain. *Proc. Natl Acad. Sci. USA* **85:** 1209–1212.

Sempe, P., Bedossa, P., Richard, M. F., Villa, M. C., Bach, J. F. and Boitard, C. (1991) Anti-α/β T cell receptor monoclonal antibody provides an efficient therapy for autoimmune diabetes in nonobese diabetic (NOD) mice. *Eur. J. Immunol.* **21:** 1163–1169.

Sempe, P., Richard, M. F., Bach, J. F. and Bostard, C. (1994) Evidence of CD4$^+$ regulatory T cells in the non-obese diabetic male mouse. *Diabetologia* **37:** 337–343.

Serreze, D. V. and Leiter, E. H. (1988) Defective activation of T suppressor cell function in nonobese diabetic mice. Potential relation to cytokine deficiencies. *J. Immunol.* **140:** 3801–3807.

Serreze, D. V., Hamaguchi, K. and Leiter, E. H. (1989) Immunostimulation circumvents diabetes in NOD/Lt mice. *J. Autoimmun.* **2:** 759–776.

Serreze, D. V., Chapman, H. D., Varnum, D. S., Hanson, M. S., Reifsnyder, P. C., Richard, S. D., Fleming, S. A., Leiter, E. H. and Shultz, L. D. (1996) B lymphocytes are essential for the initiation of T cell-mediated autoimmune diabetes: analysis of a new "speed congenic" stock of NOD.Ig mu null mice. *J. Exp. Med.* **184:** 2049–2053.

Serreze, D. V., Fleming, S. A., Chapman, H. D., Richard, S. D., Leiter, E. H. and Tisch, R. M. (1998) B lymphocytes are critical antigen-presenting cells for the initiation of T cell-mediated autoimmune diabetes in nonobese diabetic mice. *J. Immunol.* **161:** 3912–3918.

Servenius, B., Rask, L. and Peterson, P. A. (1987) Class II genes of the human major histocompatibility complex. The DOβ gene is a divergent member of the class II β gene family. *J. Biol. Chem.* **262:** 8759–8766.

Serwe, M., Reuter, G., Sponaas, A., Koch, S. and Koch, N. (1997) Both invariant chain isoforms Ii31 and Ii41 promote class II antigen presentation. *Int. Immunol.* **9:** 983–991.

Sette, A., Buus, S., Colon, S., Smith, J. A., Miles, C. and Grey, H. M. (1987) Structural characteristics of an antigen required for its interaction with Ia and recognition by T cells. *Nature* **328:** 395–399.

Sette, A., Adorini, L., Appella, E., Colon, S. M., Miles, C., Tanaka, S., Ehrhardt, C., Doria, G., Nagy, Z. A., Buus, S., *et al.* (1989a) Structural requirements for the interaction between peptide antigens and I-E^d molecules. *J. Immunol.* **143:** 3289–3294.

Sette, A., Buus, S., Appella, E., Smith, J. A., Chesnut, R., Miles, C., Colon, S. M. and Grey, H. M. (1989b) Prediction of major histocompatibility complex binding regions of protein antigens by sequence pattern analysis. *Proc. Natl Acad. Sci. USA* **86:** 3296–3300.

Sette, A., Buus, S., Colon, S., Miles, C. and Grey, H. M. (1989c) Structural analysis of peptides capable of binding to more than one Ia antigen. *J. Immunol.* **142:** 35–40.

Sette, A., Ceman, S., Kubo, R. T., Sakaguchi, K., Appella, E., Hunt, D. F., Davis, T. A., Michel, H., Shabanowitz, J., Rudersdorf, R., *et al.* (1992a) Invariant chain peptides in most HLA-DR molecules of an antigen-processing mutant. *Science* **258:** 1801–1804.

Sette, A., Southwood, S., O'Sullivan, D., Gaeta, F. C. A., Sidney, J. and Grey, H. M. (1992b) Effect of pH on MHC class II-peptide interactions. *J. Immunol.* **148:** 844–851.

Sette, A., Sidney, J., Oseroff, C., del Guercio, M. F., Southwood, S., Arrhenius, T., Powell, M. F., Colon, S. M., Gaeta, F. C. and Grey, H. M. (1993) HLA DR4w4-binding motifs illustrate the biochemical basis of degeneracy and specificity in peptide-DR interactions. *J. Immunol.* **151:** 3163–3170.

Sette, A., Southwood, S., Miller, J. and Appella, E. (1995) Binding of major histocompatibility complex class II to the invariant chain-derived peptide, CLIP, is regulated by allelic polymorphism in class II. *J. Exp. Med.* **181:** 677–683.

Shachar, I. and Flavell, R. A. (1996) Requirement for invariant chain in B cell maturation and function. *Science* **274:** 106–108.

Shachar, I., Elliott, E. A., Chasnoff, B., Grewal, I. S. and Flavell, R. A. (1995) Reconstitution of invariant chain function in transgenic mice *in vivo* by individual p31 and p41 isoforms. *Immunity* **3:** 373–383.

Sheehy, M. J. (1992) HLA and insulin-dependent diabetes. A protective perspective. *Diabetes* **41:** 123–129.

Shehadeh, N., Calcinaro, F., Bradley, B. J., Bruchlim, I., Vardi, P. and Lafferty, K. J. (1994) Effect of adjuvant therapy on development of diabetes in mouse and man. *Lancet* **343:** 706–707.

Sher, M. R., Bender, T. P. and Niederhuber, J. E. (1984) Two Ia.17-specific monoclonal antibodies detect the same epitope but do not share idiotype. *J. Immunol.* **133:** 338–344.

Sherman, M. A., Weber, D. A. and Jensen, P. E. (1995) DM enhances peptide binding to class II MHC by release of invariant chain-derived peptide. *Immunity* **3:** 197–205.

Shi, G. P., Munger, J. S., Meara, J. P., Rich, D. H. and Chapman, H. A. (1992) Molecular cloning and expression of human alveolar macrophage cathepsin S, an elastinolytic cysteine protease. *J. Biol. Chem.* **267:** 7258–7262.

Shi, G. P., Webb, A. C., Foster, K. E., Knoll, J. H., Lemere, C. A., Munger, J. S. and Chapman, H. A. (1994) Human cathepsin S: chromosomal localization, gene structure, and tissue distribution. *J. Biol. Chem.* **269:** 11530–11536.

Shi, G. P., Villadangos, J. A., Dranoff, G., Small, C., Gu, L., Haley, K. J., Riese, R., Ploegh, H. L. and Chapman, H. A. (1999) Cathepsin S required for normal MHC class II peptide loading and germinal center development. *Immunity* **10:** 197–206.

Shimada, A., Charlton, B., Rohane, P., Taylor-Edwards, C. and Fathman, C. G. (1996) Immune regulation in type 1 diabetes. *J. Autoimmun.* **9:** 263–269.

Shimizu, J., Carrasco-Marin, E., Kanagawa, O. and Unanue, E. R. (1995) Relationship between b-cell injury and antigen presentation in NOD mice. *J. Immunol.* **155:** 4095–4099.

Shimonkevitz, R., Kappler, J., Marrack, P. and Grey, H. (1983) Antigen recognition by H-2-restricted T cells. I. Cell-free antigen processing. *J. Exp. Med.* **158:** 303–316.

Shindo, Y., Inoko, H., Yamamoto, T. and Ohno, S. (1994) HLA-DRB1 typing of Vogt-Koyanagi-Harada's disease by PCR-RFLP and the strong association with DRB1*0405 and DRB1*0410. *Br. J. Ophthalmol.* **78:** 223–226.

Shizuru, J. A., Taylor-Edwards, C., Banks, B. A., Gregory, A. K. and Fathman, C. G. (1988) Immunotherapy of the nonobese diabetic mouse: treatment with an antibody to T-helper lymphocytes. *Science* **240:** 659–662.

Shortman, K., Egerton, M., Spangrude, G. J. and Scollay, R. (1990) The generation and fate of thymocytes. *Semin. Immunol.* **2:** 3–12.

Signore, A., Pozzilli, P., Gale, E. A., Andreani, D. and Beverley, P. C. (1989) The natural history of lymphocyte subsets infiltrating the pancreas of NOD mice. *Diabetologia* **32:** 282–289.

Signore, A., Annovazzi, A., Procaccini, E., Beales, P. E., Spencer, J., Testi, R. and Ruberti, G. (1997) CD95 and CD95-ligand expression in endocrine pancreas of NOD, NOR and BALB/c mice. *Diabetologia* **40:** 1476–1479.

Silver, J., Walker, L. E., Reisfeld, R. A., Pellegrino, M. A. and Ferrone, S. (1979) Structural studies of murine I-E and human DR antigens. *Mol. Immunol.* **16:** 37–42.

Silver, M. L., Guo, H. C., Strominger, J. L. and Wiley, D. C. (1992) Atomic structure of a human MHC molecule presenting an influenza virus peptide. *Nature* **360:** 367–369.

Silverman, D. and Rose, N. R. (1975) Spontaneous and methylcholanthrene-enhanced thyroiditis in BUF rats. II. Induction of experimental autoimmune thyroiditis without completed Freund's adjuvant. *J. Immunol.* **114:** 148–150.

Simonis, S. and Cullen, S. E. (1986) Fatty acylation of murine Ia α, β, and invariant chains. *J. Immunol.* **136:** 2962–2967.

Simonsen, A., Momburg, F., Drexler, J., Hämmerling, G. and Bakke, O. (1993) Intracellular distribution of the MHC class II molecules and the associated invariant chain (Ii) in different cell lines. *Int. Immunol.* **5:** 903–917.

Singer, S. J. (1990) The structure and insertion of integral proteins in membranes. *Annu. Rev. Cell Biol.* **6:** 247–296.

Singer, P. A., Lauer, W., Dembic, Z., Mayer, W. E., Lipp, J., Koch, N., Hämmerling, G., Klein, J. and Dobberstein, B. (1984) Structure of the murine Ia-associated invariant (Ii) chain as deduced from a cDNA clone. *EMBO J.* **3:** 873–877.

Singer, S. M., Tisch, R., Yang, X. D. and McDevitt, H. O. (1993) An $A\beta^d$ transgene prevents diabetes in nonobese diabetic mice by inducing regulatory T cells. *Proc. Natl Acad. Sci. USA* **90:** 9566–9570.

Singer, S. M., Umetsu, D. T. and McDevitt, H. O. (1996) High copy number I-Aβ transgenes induce production of IgE through an interleukin 4-dependent mechanism. *Proc. Natl Acad. Sci. USA* **93:** 2947–2952.

Singer, S. M., Tisch, R., Yang, X. D., Sytwu, H. K., Liblau, R. and McDevitt, H. O. (1998) Prevention of diabetes in NOD mice by a mutated I-Aβ transgene. *Diabetes* **47:** 1570–1577.

Singh, B. and Rabinovitch, A. (1993) Influence of microbial agents on the development and prevention of autoimmune diabetes. *Autoimmunity* **15:** 209–213.

Slattery, R. M. and Miller, J. F. (1996) Influence of T lymphocytes and major histocompatibility complex class II genes on diabetes susceptibility in the NOD mouse. *Curr. Top. Microbiol. Immunol.* **206:** 51–66.

Slattery, R. M., Kjer-Nielsen, L., Allison, J., Charlton, B., Mandel, T. E. and Miller, J. F. A. P. (1990) Prevention of diabetes in non-obese diabetic $I\text{-}A^k$ transgenic mice. *Nature* **345:** 724–726.

Sloan, V. S., Cameron, P., Porter, G., Gammon, M., Amaya, M., Mellins, E. and Zaller, D. M. (1995) Mediation by HLA-DM of dissociation of peptides from HLA-DR. *Nature* **375:** 802–806.

Smilek, D. E., Lock, C. B. and McDevitt, H. O. (1990) Antigen recognition and peptide-mediated immunotherapy in autoimmune disease. *Immunol. Rev.* **118:** 37–71.

Snell, G. D. (1948) Methods for the study of histocompatibility genes. *J. Genet.* **49:** 87–108.

Sollid, L. M., Markussen, G., Ek, J., Gjerde, H., Vartdal, F. and Thorsby, E. (1989) Evidence for a primary association of celiac disease to a particular HLA-DQ αβ heterodimer. *J. Exp. Med.* **169:** 345–350.

Southern, P. J. and Berg, P. (1982) Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. *J. Mol. Appl. Genet.* **1:** 327–341.

Spiro, R. C. and Quaranta, V. (1989) The invariant chain is a phosphorylated subunit of class II molecules. *J. Immunol.* **143:** 2589–2594.

Spits, H., Blom, B., Jaleco, A. C., Weijer, K., Verschuren, M. C., van Dongen, J. J., Heemskerk, M. H. and Res, P. C. (1998) Early stages in the development of human T, natural killer and thymic dendritic cells. *Immunol. Rev.* **165:** 75–86.

Springer, T. A., Kaufman, J. F., Siddoway, L. A., Mann, D. L. and Strominger, J. L. (1977) Purification of *HLA*-linked B lymphocyte alloantigens in immunologically active form by preparative sodium dodecyl sulfate-gel electrophoresis and studies on their subunit association. *J. Biol. Chem.* **252:** 6201–6207.

Spurkland, A., Gilhus, N. E., Ronningen, K. S., Aarli, J. A. and Vartdal, F. (1991) Myasthenia gravis patients with thymus hyperplasia and myasthenia gravis patients with thymoma display different HLA associations. *Tissue Antigens* **37:** 90–93.

Srikanta, S., Ganda, O. P., Rabizadeh, A., Soeldner, J. S. and Eisenbarth, G. S. (1985) First-degree relatives of patients with type I diabetes mellitus. Islet-cell antibodies and abnormal insulin secretion. *N. Engl. J. Med.* **313:** 461–464.

Srinivasan, M., Marsh, E. W. and Pierce, S. K. (1991) Characterisation of naturally-processed antigen bound to major histocompatibility complex class II molecules. *Proc. Natl Acad. Sci. USA* **88:** 7928–7932.

Stang, E., Guerra, C. B., Amaya, M., Paterson, Y., Bakke, O. and Mellins, E. D. (1998) DR/CLIP (class II-associated invariant chain peptides) and DR/peptide complexes colocalise in prelysosomes in human B lymphoblastoid cells. *J. Immunol.* **160:** 4696–4707.

Stassi, G., De Maria, R., Trucco, G., Rudert, W., Testi, R., Galluzzo, A., Giordano, C. and Trucco, M. (1997) Nitric oxide primes pancreatic β cells for Fas-mediated destruction in insulin-dependent diabetes mellitus. *J. Exp. Med.* **186:** 1193–1200.

Stebbins, C. C., Loss, G. E. J., Elias, C. G., Chervonsky, A. and Sant, A. J. (1995) The requirement for DM in class II-restricted antigen presentation and SDS-stable dimer formation is allele and species dependent. *J. Exp. Med.* **181:** 223–234.

Stebbins, C. C., Peterson, M. E., Suh, W. M. and Sant, A. J. (1996) DM-mediated release of a naturally-occurring invariant chain degradation intermediate from MHC class II molecules. *J. Immunol.* **157:** 4892–4898.

Steinman, L. (1995) Escape from "horror autotoxicus": pathogenesis and treatment of autoimmune disease. *Cell* **80:** 7–10.

Sterkers, G., Zeliszewski, D., Chaussee, A. M., Deschamps, I., Font, M. P., Freidel, C., Hors, J., Betuel, H., Dausset, J. and Levy, J. P. (1988) HLA-DQ rather than HLA-DR region might be involved in dominant nonsusceptibility to diabetes. *Proc. Natl Acad. Sci. USA* **85:** 6473–6477.

Stern, L. J., Brown, J. H., Jardetzky, T. S., Gorga, J. C., Urban, R. G., Strominger, J. L. and Wiley, D. C. (1994) Crystal structure of the human class II MHC protein HLA-DR1 complexed with an influenza virus peptide. *Nature* **368:** 215–221.

Sternthal, E., Like, A. A., Sarantis, K. and Braverman, L. E. (1981) Lymphocytic thyroiditis and diabetes in the BB/W rat. A new model of autoimmune endocrinopathy. *Diabetes* **30:** 1058–1061.

Stiller, C. R., Dupre, J., Gent, M., Jenner, M. R., Keown, P. A., Laupacis, A., Martell, R., Rodger, N. W., von Graffenried, B. and Wolfe, B. M. (1984) Effects of cyclosporine immunosuppression in insulin-dependent diabetes mellitus of recent onset. *Science* **223:** 1362–1367.

Streicher, H. Z., Berkower, I. J., Busch, M., Gurd, F. R. N. and Berzofsky, J. A. (1984) Antigen conformation determines processing requirements for T-cell activation. *Proc. Natl Acad. Sci. USA* **81:** 6831–6835.

Strubin, M., Mach, B. and Long, E. O. (1984) The complete sequence of the mRNA for the HLA-DR-associated invariant chain reveals a polypeptide with an unusual transmembrane polarity. *EMBO J.* **3:** 869–872.

Strubin, M., Berte, C. and Mach, B. (1986a) Alternative splicing and alternative initiation of translation explain the four forms of the Ia antigen-associated invariant chain. *EMBO J.* **5:** 3483–3488.

Strubin, M., Long, E. O. and Mach, B. (1986b) Two forms of the Ia antigen-associated invariant chain result from alternative initiations at two in-phase AUGs. *Cell* **47:** 619–625.

Stumptner, P. and Benaroch, P. (1997) Interaction of MHC class II molecules with the invariant chain: Role of the invariant chain (81–90) region. *EMBO J.* **16:** 5807–5818.

Sugita, M. and Brenner, M. B. (1995) Association of the invariant chain with major histocompatibility complex class I molecules directs trafficking to endocytic compartments. *J. Biol. Chem.* **270:** 1443–1448.

Sung, E. and Jones, P. P. (1981) The invariant chain of murine Ia antigens: its glycosylation, abundance and subcellular localization. *Mol. Immunol.* **18:** 899–913.

Surh, C. D., Lee, D. S., Fung-Leung, W. P., Karlsson, L. and Sprent, J. (1997) Thymic selection by a single MHC/peptide ligand produces a semidiverse repertoire of CD4$^+$ T cells. *Immunity* **7:** 209–219.

Swier, K. and Miller, J. (1995a) Efficient internalisation of MHC class II-invariant chain complexes is not sufficient for invariant chain proteolysis and class II antigen presentation. *J. Immunol.* **155:** 630–643.

Swier, K. and Miller, J. (1995b) Invariant chain-independent antigen presentation depends primarily upon the pool of newly-synthesised MHC class II molecules. *J. Immunol.* **155:** 1851–1861.

Sykulev, Y., Brunmark, A., Jackson, M., Cohen, R. J., Peterson, P. A. and Eisen, H. N. (1994) Kinetics and affinity of reactions between an antigen-specific T cell receptor and peptide-MHC complexes. *Immunity* **1:** 15–22.

Takaesu, N. T., Lower, J. A., Robertson, E. J. and Bikoff, E. K. (1995) Major histocompatibility class II peptide occupancy, antigen presentation, and CD4$^+$ T cell function in mice lacking the p41 isoform of invariant chain. *Immunity* **3:** 385–396.

Takaesu, N. T., Lower, J. A., Yelon, D., Robertson, E. J. and Bikoff, E. K. (1997) *In vivo* functions mediated by the p41 isoform of the MHC class II associated invariant chain. *J. Immunol.* **158:** 187–199.

Tampé, R. and McConnell, H. M. (1991) Kinetics of antigenic peptide binding to the class II major histocompatibility molecule, I-A^d. *Proc. Natl Acad. Sci. USA* **88:** 4661–4665.

Tan, P. L., Farmiloe, S., Roberts, M., Geursen, A. and Skinner, M. A. (1993) HLA-DR4 subtypes in New Zealand Polynesians. Predominance of Dw13 in the healthy population and association of Dw15 with rheumatoid arthritis. *Arthritis Rheum.* **36:** 15–19.

Tan, L. J., Ceman, S., Chervonsky, A., Rodriguez-Paris, J., Steck, T. L. and Sant, A. J. (1997a) Late events in the intracellular sorting of major histocompatibility complex class II molecules are regulated by the 80–82 segment of the class II β-chain. *Eur. J. Immunol.* **27:** 1479–1488.

Tan, M. C., Mommaas, A. M., Drijfhout, J. W., Jordens, R., Onderwater, J. J., Verwoerd, D., Mulder, A. A., van der Heiden, A. N., Scheidegger, D., Oomen, L. C., *et al.* (1997b) Mannose receptor-mediated uptake of antigens strongly enhances HLA class II-restricted antigen presentation by cultured dendritic cells. *Eur. J. Immunol.* **27:** 2426–2435.

Tate, K. M., Lee, C., Edelman, S., Carswell-Crumpton, C., Liblau, R. and Jones, P. P. (1995) Interactions among polymorphic and conserved residues in MHC class II proteins affect MHC-peptide conformation and T cell recognition. *Int. Immunol.* **7:** 747–761.

Teh, H. S., Kisielow, P., Scott, B., Kishi, H., Uematsu, Y., Bluthmann, H. and von Boehmer, H. (1988) Thymic major histocompatibility complex antigens and the αβ T-cell receptor determine the CD4/CD8 phenotype of T cells. *Nature* **335:** 229–233.

ten Bosch, G. J., Kessler, J. H., Joosten, A. M., Bres-Vloemans, A. A., Geluk, A., Godthelp, B. C., van Bergen, J., Melief, C. J. and Leeksma, O. C. (1999) A BCR-ABL oncoprotein p210b2a2 fusion region sequence is recognized by HLA-DR2a restricted cytotoxic T lymphocytes and presented by HLA-DR matched cells transfected with an Ii^{b2a2} construct. *Blood* **94:** 1038–1045.

Teyton, L., O'Sullivan, D., Dickson, P. W., Lotteau, V., Sette, A., Fink, P. and Peterson, P. A. (1990) Invariant chain distinguishes between the exogenous and endogenous antigen presentation pathways. *Nature* **348:** 39–44.

Thomas-Vaslin, V., Damotte, D., Coltey, M., Le Douarin, N. M., Coutinho, A. and Salaun, J. (1997) Abnormal T cell selection on nod thymic epithelium is sufficient to induce autoimmune manifestations in C57BL/6 athymic nude mice. *Proc. Natl Acad. Sci. USA* **94:** 4598–4603.

Thorsby, E. (1995) HLA-associated disease susceptibility — Which genes are primarily involved? *The Immunologist* **3:** 51–58.

Thorsby, E. and Rønningen, K. S. (1993) Particular HLA-DQ molecules play a dominant role in determining susceptibility or resistance to type 1 (insulin-dependent) diabetes mellitus. *Diabetologia* **36:** 371–377.

Tian, J., Lehmann, P. V. and Kaufman, D. L. (1994) T cell cross-reactivity between coxsackie virus and glutamate decarboxylase is associated with a murine diabetes susceptibility allele. *J. Exp. Med.* **180:** 1979–1984.

Tian, J., Atkinson, M. A., Clare-Salzler, M., Herschenfeld, A., Forsthuber, T., Lehmann, P. V. and Kaufman, D. L. (1996) Nasal administration of glutamate decarboxylase (GAD65) peptides induces Th2 responses and prevents murine insulin-dependent diabetes. *J. Exp. Med.* **183:** 1561–1567.

Tisch, R., Yang, X.-D., Singer, S. M., Liblau, R. S., Fugger, L. and McDevitt, H. O. (1993) Immune response to glutamic acid decarboxylase correlates with insulitis in non-obese diabetic mice. *Nature* **366:** 72–75.

Tisch, R., Yang, X. D., Liblau, R. S. and McDevitt, H. O. (1994) Administering glutamic acid decarboxylase to NOD mice prevents diabetes. *J. Autoimmun.* **7:** 845–850.

Todd, J. A., Bell, J. I. and McDevitt, H. O. (1987) HLA-DQβ gene contributes to susceptibility and resistance to insulin-dependent diabetes mellitus. *Nature* **368:** 599–602.

Tonnelle, C., DeMars, R. and Long, E. O. (1985) DOβ: a new β-chain gene in HLA-D with a distinct regulation of expression. *EMBO J.* **4:** 2839–2847.

Tourne, S., Nakano, N., Viville, S., Benoist, C. and Mathis, D. (1995) The influence of invariant chain on the positive selection of single T cell receptor specificities. *Eur. J. Immunol.* **25:** 1851–1856.

Tourne, S., Miyazaki, T., Oxenius, A., Klein, L., Fehr, T., Kyewski, B., Benoist, C. and Mathis, D. (1997) Selection of a broad repertoire of CD4$^+$ T cells in H-2Ma$^{0/0}$ mice. *Immunity* **7:** 187–195.

Townsend, A., Ohlen, C., Bastin, J., Ljunggren, H. G., Foster, L. and Karre, K. (1989) Association of class I major histocompatibility heavy and light chains induced by viral peptides. *Nature* **340:** 443–448.

Toyota, T., Satoh, J., Oya, K., Shintani, S. and Okano, T. (1986) Streptococcal preparation (OK-432) inhibits development of type I diabetes in NOD mice. *Diabetes* **35:** 496–499.

Trowsdale, J. and Kelly, A. (1985) The human HLA class II α-chain gene *DZa* is distinct from genes in the DP, DQ and DR subregions. *EMBO J.* **4:** 2231–2237.

Trucco, M., de Petris, S., Garotta, G. and Ceppellini, R. (1980) Quantitative analysis of cell-surface HLA structures by means of monoclonal antibodies. *Hum. Immunol.* **1:** 233–243.

Tulp, A., Verwoerd, D., Dobberstein, B., Ploegh, H. L. and Pieters, J. (1994) Isolation and characterisation of the intracellular MHC class II compartment. *Nature* **369:** 120–126.

Uchigata, Y., Kuwata, S., Tokunaga, K., Eguchi, Y., Takayama-Hasumi, S., Miyamoto, M., Omori, Y., Juji, T. and Hirata, Y. (1992) Strong association of insulin autoimmune syndrome with HLA-DR4. *Lancet* **339:** 393–394.

Uehira, M., Uno, M., Kurner, T., Kikutani, H., Mori, K., Inomoto, T., Uede, T., Miyazaki, J., Nishimoto, H., Kishimoto, T., *et al.* (1989) Development of autoimmune insulitis is prevented in Eα^d but not in Aβ^k NOD transgenic mice. *Int. Immunol.* **1:** 209–213.

Ullrich, H. J., Doring, K., Gruneberg, U., Jahnig, F., Trowsdale, J. and van Ham, S. M. (1997) Interaction between HLA-DM and HLA-DR involves regions that undergo conformational changes at lysosomal pH. *Proc. Natl Acad. Sci. USA* **94:** 13163–13168.

Undlien, D. E., Friede, T., Rammensee, H. G., Joner, G., Dahl-Jørgensen, K., Søvik, O., Akselsen, H. E., Knutsen, I., Rønningen, K. S. and Thorsby, E. (1997) HLA-encoded genetic predisposition in IDDM: DR4 subtypes may be associated with different degrees of protection. *Diabetes* **46:** 143–149.

Urban, R. G., Chicz, R. M. and Strominger, J. L. (1994) Selective release of some invariant chain-derived peptides from HLA-DR1 molecules at endosomal pH. *J. Exp. Med.* **180:** 751–755.

Utsugi, T., Yoon, J. W., Park, B. J., Imamura, M., Averill, N., Kawazu, S. and Santamaria, P. (1996) Major histocompatibility complex class I restricted infiltration and destruction of pancreatic islets by NOD mouse-derived β-cell cytotoxic CD8$^+$ T-cell clones in vivo. *Diabetes* **45:** 1121–1131.

Vague, P., Vialettes, B., Lassmann-Vague, V. and Vallo, J. J. (1987) Nicotinamide may extend remission phase in insulin-dependent diabetes. *Lancet* **1:** 619–620.

Valitutti, S. and Lanzavecchia, A. (1997) Serial triggering of TCRs: A basis for the sensitivity and specificity of antigen recognition. *Immunol. Today* **18:** 299–304.

Valitutti, S., Müller, S., Cella, M., Padovan, E. and Lanzavecchia, A. (1995) Serial triggering of many T-cell receptors by a few peptide-MHC complexes. *Nature* **375:** 148–151.

van Bergen, J., Schoenberger, S. P., Verreck, F., Amons, R., Offringa, R. and Koning, F. (1997) Efficient loading of HLA-DR with a T helper epitope by genetic exchange of CLIP. *Proc. Natl Acad. Sci. USA* **94:** 7499–7502.

van de Wal, Y., Kooy, Y. M. C., Drijfhout, J. W., Amons, R. and Koning, F. (1996) Peptide binding characteristics of the coeliac disease-associated DQ(α1*0501, β1*0201) molecule. *Immunogenetics* **44:** 246–253.

van de Wal, Y., Kooy, Y. M., Drijfhout, J. W., Amons, R., Papadopoulos, G. K. and Koning, F. (1997) Unique peptide binding characteristics of the disease-associated DQ(α1*0501, β1*0201) vs the non-disease-associated DQ(α1*0201, β1*0202) molecule. *Immunogenetics* **46:** 484–492.

van der Auwera, B. J., Heimberg, H., Schrevens, A. F., van Waeyenberge, C., Flament, J. and Schuit, F. C. (1993) 5' insulin gene polymorphism confers risk to IDDM independently of HLA class II susceptibility. *Diabetes* **42:** 851–854.

van Ham, S. M., Grüneberg, U., Malcherek, G., Bröker, I., Melms, A. and Trowsdale, J. (1996) Human histocompatibility leukocyte antigen (HLA)-DM edits peptides presented by HLA-DR according to their ligand binding motifs. *J. Exp. Med.* **184:** 2019–2024.

van Ham, S. M., Tjin, E. P. M., Lillemeier, B. F., Gruneberg, U., van Meijgaarden, K. E., Pastoors, L., Verwoerd, D., Tulp, A., Canas, B., Rahman, D., *et al.* (1997) HLA-DO is a negative modulator of HLA-DM-mediated MHC class II peptide loading. *Curr. Biol.* **7:** 950–957.

Vartdal, F., Johansen, B. H., Friede, T., Thorpe, C. J., Stevanovic, S., Eriksen, J. E., Sletten, K., Thorsby, E., Rammensee, H.-G. and Sollid, L. M. (1996) The peptide binding motif of the disease associated HLA-DQ (α1*0501, β1*0201) molecule. *Eur. J. Immunol.* **26:** 2764–2772.

Vath, G. M., Earhart, C. A., Rago, J. V., Kim, M. H., Bohach, G. A., Schlievert, P. M. and Ohlendorf, D. H. (1997) The structure of the superantigen exfoliative toxin A suggests a novel regulation as a serine protease. *Biochemistry* **36:** 1559–1566.

Vaysburd, M., Lock, C. and McDevitt, H. (1995) Prevention of insulin-dependent diabetes mellitus in nonobese diabetic mice by immunogenic but not by tolerated peptides. *J. Exp. Med.* **182:** 897–902.

Veenstra, H., Jacobs, P. and Dowdle, E. B. (1993) Processing of HLA-class II invariant chain and expression of the p35 form is different in malignant and transformed cells. *Blood* **82:** 2494–2500.

Vergelli, M., Pinet, V., Vogt, A. B., Kalbus, M., Malnati, M., Riccio, P., Long, E. O. and Martin, R. (1997) HLA-DR-restricted presentation of purified myelin basic protein is independent of intracellular processing. *Eur. J. Immunol.* **27:** 941–951.

Verreck, F. A., Termijtelen, A. and Koning, F. (1993) HLA-DR β-chain reside 86 controls DR αβ dimer stability. *Eur. J. Immunol.* **23:** 1346–1350.

Vigna, J. L., Smith, K. D. and Lutz, C. T. (1996) Invariant chain association with MHC class I: preference for HLA class I/β_2-microglobulin heterodimers, specificity, and influence of the MHC peptide-binding groove. *J. Immunol.* **157:** 4503–4510.

Villadangos, J. A., Riese, R. J., Peters, C., Chapman, H. A. and Ploegh, H. L. (1997) Degradation of mouse invariant chain: roles of cathepsins S and D and the influence of major histocompatibility complex polymorphism. *J. Exp. Med.* **186:** 549–560.

Viola, A. and Lanzavecchia, A. (1996) T cell activation determined by T cell receptor number and tunable thresholds. *Science* **273:** 104–106.

Viville, S., Neefjes, J., Lotteau, V., Dierich, A., Lemeur, M., Ploegh, H., Benoist, C. and Mathis, D. (1993) Mice lacking the MHC class II-associated invariant chain. *Cell* **72:** 635–648.

Vladutiu, A. O. and Rose, N. R. (1971) Autoimmune murine thyroiditis relation to histocompatibility (H-2) type. *Science* **174:** 1137–1139.

Vogt, A. B., Stern, L. J., Amshoff, C., Dobberstein, B., Hämmerling, G. J. and Kropshofer, H. (1995) Interference of distinct invariant chain regions with superantigen contact area and antigenic peptide binding groove of HLA-DR. *J. Immunol.* **155:** 4757–4765.

Vogt, A. B., Kropshofer, H., Moldenhauer, G. and Hämmerling, G. J. (1996) Kinetic analysis of peptide loading onto HLA-DR molecules mediated by HLA-DM. *Proc. Natl Acad. Sci. USA* **93:** 9724–9729.

von Bonin, A., Ehrlich, S., Malcherek, G. and Fleischer, B. (1995) Major histocompatibility complex class II-associated peptides determine the binding of the superantigen toxic shock syndrome toxin-1. *Eur. J. Immunol.* **25:** 2894–2898.

Wacker, W. B. and Kalsow, C. M. (1973) Autoimmune uveo-retinitis in the rat sensitised with retina photoreceptor cell antigen. *Int. Arch. Allergy Appl. Immunol.* **45:** 582–592.

Wade, J. A. (1996) New molecular approaches to tissue typing. *Transplant. Proc.* **28:** 2022–2023.

Wake, C. T. and Flavell, R. A. (1985) Multiple mechanisms regulate the expression of murine immune response genes. *Cell* **42:** 623–628.

Wang, B., Gonzalez, A., Benoist, C. and Mathis, D. (1996) The role of $CD8^+$ T cells in the initiation of insulin-dependent diabetes mellitus. *Eur. J. Immunol.* **26:** 1762–1769.

Wang, K., Peterson, P. A. and Karlsson, L. (1997) Decreased endosomal delivery of major histocompatibility complex class II-invariant chain complexes in dynamin-deficient cells. *J. Biol. Chem.* **272:** 17055–17060.

Watson, J. (1979) Continous proliferation of murine antigen-specific helper T lymphocytes in culture. *J. Exp. Med.* **150:** 1510–1519.

Watts, C. (1997) Capture and processing of exogenous antigens for presentation on MHC molecules. *Annu. Rev. Immunol.* **15:** 821–850.

Watts, T. H. and McConnell, H. M. (1986) High-affinity fluorescent peptide binding to I-Ad in lipid membranes. *Proc. Natl Acad. Sci. USA* **83:** 9660–9664.

Wauben, M. H. M., van der Kraan, M., Grosfeld-Stulemeyer, M. C. and Joosten, I. (1997) Definition of an extended MHC class II-peptide binding motif for the autoimmune disease-associated Lewis rat $RT1.B^l$ molecule. *Int. Immunol.* **9:** 281–290.

Weber, D. A., Evavold, B. D. and Jensen, P. E. (1996) Enhanced dissociation of HLA-DR-bound peptides in the presence of HLA-DM. *Science* **274:** 618–621.

Weber, P., Raynaud, I., Ettouati, L., Trescol-Biémont, M. C., Carrupt, P. A., Paris, J., Rabourdin-Combe, C., Gerlier, D. and Testa, B. (1998) Molecular modeling of hen egg lysozyme HEL[52–61] peptide binding to $I\text{-}A^k$ MHC class II molecule. *Int. Immunol.* **10:** 1753–1764.

Weenink, S. M., Averdunk, H., Boston, T., Boswarva, V., Guery, J.-C., Adorini, L., Mellins, E., McCluskey, J. and Gautam, A. M. (1997) Impaired antigen presentation by murine $I\text{-}A^d$ class II

MHC molecules expressed in normal and HLA-DM-defective human B cell lines. *Int. Immunol.* **9:** 889–896.

Wegmann, D. R. (1996) The immune response to islets in experimental diabetes and insulin-dependent diabetes mellitus. *Curr. Op. Immunol.* **8:** 860–864.

Wegmann, D. R., Norbury-Glaser, M. and Daniel, D. (1994) Insulin-specific T cells are a predominant component of islet infiltrates in pre-diabetic NOD mice. *Eur. J. Immunol.* **24:** 1853–1857.

Weiss, A. (1993) T cell antigen receptor signal transduction: a tale of tails and cytoplasmic protein-tyrosine kinases. *Cell* **73:** 209–212.

Weiss, S. and Bogen, B. (1991) MHC class II-restricted presentation of intracellular antigen. *Cell* **64:** 767–776.

Wen, R., Cole, G. A., Surman, S., Blackman, M. A. and Woodland, D. L. (1996) Major histocompatibility complex class II-associated peptides control the presentation of bacterial superantigens to T cells. *J. Exp. Med.* **183:** 1083–1092.

Wen, R., Broussard, D. R., Surman, S., Hogg, T. L., Blackman, M. A. and Woodland, D. L. (1997) Carboxy-terminal residues of major histocompatibility complex class II- associated peptides control the presentation of the bacterial superantigen toxic shock syndrome toxin-1 to T cells. *Eur. J. Immunol.* **27:** 772–781.

West, M. A., Lucocq, J. M. and Watts, C. (1994) Antigen processing and class II MHC peptide-loading compartments in human lymphoblastoid cells. *Nature* **369:** 147–151.

Wettstein, D. A., Boniface, J. J., Reay, P. A., Schild, H. and Davis, M. M. (1991) Expression of a class II major histocompatibility complex (MHC) heterodimer in a lipid-linked form with enhanced peptide/soluble MHC complex formation at low pH. *J. Exp. Med.* **174:** 219–228.

Wherrett, D. K., Singer, S. M. and McDevitt, H. O. (1997) Reduction in diabetes incidence in an I-A^{g7} transgenic nonobese diabetic mouse line. *Diabetes* **46:** 1970–1974.

White, J., Herman, A., Pullen, A. M., Kubo, R., Kappler, J. W. and Marrack, P. (1989) The Vβ-specific superantigen staphylococcal enterotoxin B: stimulation of mature T cells and clonal deletion in neonatal mice. *Cell* **56:** 27–35.

Wicker, L. S., Miller, B. J. and Mullen, Y. (1986) Transfer of autoimmune diabetes mellitus with splenocytes from nonobese diabetic (NOD) mice. *Diabetes* **35:** 855–860.

Wicker, L. S., Miller, B. J., Fischer, P. A., Pressey, A. and Peterson, L. B. (1989) Genetic control of diabetes and insulitis in the nonobese diabetic mouse. Pedigree analysis of a diabetic H-$2^{nod/b}$ heterozygote. *J. Immunol.* **142:** 781–784.

Wicker, L. S., Appel, M. C., Dotta, F., Pressey, A., Miller, B. J., DeLarato, N. H., Fischer, P. A., Boltz, R. C., Jr. and Peterson, L. B. (1992) Autoimmune syndromes in major histocompatibility complex (MHC) congenic strains of nonobese diabetic (NOD) mice. The NOD MHC is dominant for insulitis and cyclophosphamide-induced diabetes. *J. Exp. Med.* **176:** 67–77.

Wicker, L. S., Todd, J. A. and Peterson, L. B. (1995) Genetic control of autoimmune diabetes in the NOD mouse. *Annu. Rev. Immunol.* **13:** 179–200.

Widera, G. and Flavell, R. A. (1985) The I region of the C57BL/10 mouse: characterisation and physical linkage to H-2K of an SB β-like class II pseudogene, φAβ3. *Proc. Natl Acad. Sci. USA* **82:** 5500–5504.

Wilberz, S., Partke, H. J., Dagnaes-Hansen, F. and Herberg, L. (1991) Persistent MHV (mouse hepatitis virus) infection reduces the incidence of diabetes mellitus in non-obese diabetic mice. *Diabetologia* **34:** 2–5.

Williams, M. A. and Fukuda, M. (1990) Accumulation of membrane glycoproteins in lysosomes requires a tyrosine residue at a particular position in the cytoplasmic tail. *J. Cell Biol.* **111:** 955–966.

Williams, D. B. and Watts, T. H. (1995) Molecular chaperones in antigen presentation. *Curr. Opin. Immunol.* **7:** 77–84.

Wilson, I. A. (1996) Another twist to MHC–peptide recognition. *Science* **272:** 973–974.

Witt, S. N. and McConnell, H. M. (1991) A first-order reaction controls the binding of antigenic peptides to major histocompatibility complex class II molecules. *Proc. Natl Acad. Sci. USA* **88:** 8164–8168.

Wolf, E., Spencer, K. M. and Cudworth, A. G. (1983) The genetic susceptibility to type 1 (insulin-dependent) diabetes: analysis of the HLA-DR association. *Diabetologia* **24:** 224–230.

Wong, F. S. and Janeway, C. A., Jr. (1997) The role of CD4 and CD8 T cells in type 1 diabetes in the NOD mouse. *Res. Immunol.* **148:** 327–332.

Wong, P. and Rudensky, A. Y. (1996) Phenotype and function of CD4$^+$ T cells in mice lacking invariant chain. *J. Immunol.* **156:** 2133–2142.

Wong, F. S., Visintin, I., Wen, L., Granata, J., Flavell, R. and Janeway, C. A. (1998) The role of lymphocyte subsets in accelerated diabetes in nonobese diabetic-rat insulin promoter-B7-1 (NOD-RIP-B7-1) mice. *J. Exp. Med.* **187:** 1985–1993.

Wraith, D. C., Smilek, D. E., Mitchell, D. J., Steinman, L. and McDevitt, H. O. (1989) Antigen recognition in autoimmune encephalomyelitis and the potential for peptide-mediated immunotherapy. *Cell* **59:** 247–255.

Wright, J. R., Jr., Lacy, P. E., Unanue, E. R., Muszynski, C. and Hauptfeld, V. (1986) Interferon-mediated induction of Ia antigen expression on isolated murine whole islets and dispersed islet cells. *Diabetes* **35:** 1174–1177.

Wright, R. J., Bikoff, E. K. and Stockinger, B. (1998) The Ii41 isoform of invariant chain mediates both positive and negative selection events in T-cell receptor transgenic mice. *Immunology* **95:** 309–313.

Wu, S. and Gorski, J. (1996) The MHC class II-associated invariant chain-derived peptide CLIP binds to the peptide-binding groove of class II molecules. *Mol. Immunol.* **33:** 371–377.

Wu, S., Gorski, J., Eckels, D. D. and Newton-Nash, D. K. (1996) T cell recognition of MHC class II-associated peptides is independent of peptide affinity for MHC and sodium dodecyl sulphate stability of the peptide/MHC complex. Effects of conservative amino acid substitutions at anchor position 1 of influenza matrix protein, 19–31. *J. Immunol.* **156:** 3815–3820.

Wubbolts, R., Fernandez-Borja, M., Oomen, L., Verwoerd, D., Janssen, H., Calafat, J., Tulp, A., Dusseljee, S. and Neefjes, J. (1996) Direct vesicular transport of MHC class II molecules from lysosomal structures to the cell surface. *J. Cell Biol.* **135:** 611–622.

Wucherpfennig, K. W. and Strominger, J. L. (1995) Selective binding of self peptides to disease-associated major histocompatibility complex (MHC) molecules: a mechanism for MHC-linked susceptibility to human autoimmune diseases. *J. Exp. Med.* **181:** 1597–1601.

Xu, M., Capraro, G. A., Diabata, M., Reyes, V. E. and Humphreys, R. E. (1994) Cathepsin B cleavage and release of invariant chain from MHC class II molecules follow a staged pattern. *Mol. Immunol.* **31:** 723–731.

Xu, X., Song, W., Cho, H., Qiu, Y. and Pierce, S. K. (1995) Intracellular transport of invariant chain-MHC class II complexes to the peptide-loading compartment. *J. Immunol.* **155:** 2984–2992.

Yamada, K., Nonaka, K., Hanafusa, T., Miyazaki, A., Toyoshima, H. and Tarui, S. (1982) Preventive and therapeutic effects of large-dose nicotinamide injections on diabetes associated with insulitis. An observation in nonobese diabetic (NOD) mice. *Diabetes* **31:** 749–753.

Yamamoto, K., Floyd-Smith, G., Francke, U., Koch, N., Lauer, W., Dobberstein, B., Schafer, R. and Hämmerling, G. J. (1985) The gene encoding the Ia-associated invariant chain is located on chromosome 18 in the mouse. *Immunogenetics* **21:** 83–90.

Yamamura, T., Namikawa, T., Endoh, M., Kunishita, T. and Tabira, T. (1986) Experimental allergic encephalomyelitis induced by proteolipid apoprotein in Lewis rats. *J. Neuroimmunol.* **12:** 143–153.

Yanagi, K., Ishimaru, N., Haneji, N., Saegusa, K., Saito, I. and Hayashi, Y. (1998) Anti-120-kDa α-fodrin immune response with Th1-cytokine profile in the NOD mouse model of Sjögren's syndrome. *Eur. J. Immunol.* **28:** 3336–3345.

Yang, X. D., Karin, N., Tisch, R., Steinman, L. and McDevitt, H. O. (1993) Inhibition of insulitis and prevention of diabetes in nonobese diabetic mice by blocking L-selectin and very late antigen 4 adhesion receptors. *Proc. Natl Acad. Sci. USA* **90:** 10494–10498.

Yang, Y., Charlton, B., Shimada, A., Dal Canto, R. and Fathman, C. G. (1996) Monoclonal T cells identified in early NOD islet infiltrates. *Immunity* **4:** 189–194.

Yang, M., Charlton, B. and Gautam, A. M. (1997) Development of insulitis and diabetes in B cell-deficient NOD mice. *J. Autoimmun.* **10:** 257–260.

Yao, X. R. and Humphreys, R. E. (1988) Decreased expression of Ii-derived p25 and of P67/69 in hairy leukemic cells. *Leuk. Res.* **12:** 787–794.

Yassine-Diab, B., Carmichael, P., L'Faqihi, F. E., Lombardi, G., Deacock, S., de Préval, C., Coppin, H. and Lechler, R. I. (1999) Biased T-cell receptor usage is associated with allelic variation in the MHC class II peptide binding groove. *Immunogenetics* **49:** 532–540.

Yoon, J. W., Austin, M., Onodera, T. and Notkins, A. L. (1979) Isolation of a virus from the pancreas of a child with diabetic ketoacidosis. *N. Engl. J. Med.* **300:** 1173–1179.

Yoon, J. W., Yoon, C. S., Lim, H. W., Huang, Q. Q., Kang, Y., Pyun, K. H., Hirasawa, K., Sherwin, R. S. and Jun, H. S. (1999) Control of autoimmune diabetes in NOD mice by GAD expression or suppression in β cells. *Science* **284:** 1183–1187.

Zamvil, S. M., Mitchell, D. J., Moore, A. C., Kitamura, K., Steinman, L. and Rothbard, J. B. (1986) T-cell epitope of the autoantigen myelin basic protein that induces encephalomyelitis. *Nature* **324:** 258–260.

Zamvil, S. S., Mitchell, D. J., Lee, N. E., Moore, A. C., Waldor, M. K., Sakai, K., Rothbard, J. B., McDevitt, H. O., Steinman, L. and Acha-Orbea, H. (1988) Predominant expression of a T cell receptor Vβ gene subfamily in autoimmune encephalomyelitis. *J. Exp. Med.* **167:** 1586–1596.

Zechel, M. A., Chaturvedi, P. and Singh, B. (1997) Characterisation of immunodominant peptide determinants of IDDM-associated autoantigens in the NOD mouse. *Res. Immunol.* **148:** 338–348.

Zechel, M. A., Krawetz, M. D. and Singh, B. (1998) Epitope dominance: evidence for reciprocal determinant spreading to glutamic acid decarboxylase in non-obese diabetic mice. *Immunol. Rev.* **164:** 111–118.

Zekzer, D., Wong, F. S., Ayalon, O., Millet, I., Altieri, M., Shintani, S., Solimena, M. and Sherwin, R. S. (1998) GAD-reactive CD4+ Th1 cells induce diabetes in NOD/SCID mice. *J. Clin. Invest.* **101:** 68–73.

Zhang, Z. J., Davidson, L., Eisenbarth, G. and Weiner, H. L. (1991) Suppression of diabetes in nonobese diabetic mice by oral administration of porcine insulin. *Proc. Natl Acad.Sci. USA* **88:** 10252–10256.

Zhong, G., Romagnoli, P. and Germain, R. N. (1997) Related leucine-based cytoplasmic targeting signals in invariant chain and major histocompatibility complex class II molecules control endocytic presentation of distinct determinants in a single protein. *J. Exp. Med.* **185:** 429–438.

Ziegler, H. K. and Unanue, E. R. (1982) Decrease in macrophage antigen catabolism caused by ammonia and chloroquine is associated with inhibition of antigen presentation to T cells. *Proc. Natl Acad. Sci. USA* **79:** 175–178.

Zimmet, P. Z., Elliott, R. B., Mackay, I. R., Tuomi, T., Rowley, M. J., Pilcher, C. C. and Knowles, W. J. (1994) Autoantibodies to glutamic acid decarboxylase and insulin in islet cell antibody positive presymptomatic type 1 diabetes mellitus: frequency and segregation by age and gender. *Diabet. Med.* **11:** 866–871.

Zinkernagel, R. M. and Doherty, P. C. (1974) Restriction of in vitro T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. *Nature* **248:** 701–702.

Zipris, D., Crow, A. R. and Delovitch, T. L. (1991a) Altered thymic and peripheral T-lymphocyte repertoire preceding onset of diabetes in NOD mice. *Diabetes* **40:** 429–435.

Zipris, D., Lazarus, A. H., Crow, A. R., Hadzija, M. and Delovitch, T. L. (1991b) Defective thymic T cell activation by concanavalin A and anti-CD3 in autoimmune nonobese diabetic mice. Evidence for thymic T cell anergy that correlates with the onset of insulitis. *J. Immunol.* **146:** 3763–3771.

Zuniga-Pflücker, J. C. and Lenardo, M. J. (1996) Regulation of thymocyte development from immature progenitors. *Curr. Opin. Immunol.* **8:** 215–224.